Exploration of the Molecular Genetics of Exudative Age-related Macular Degeneration

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Abstract

Age-related macular degeneration (AMD) is a leading cause of severe visual impairment affecting about 1.5% of most populations worldwide. It is a late-onset, chronic and progressive degenerative disorder involving typical lesions in the macular and choroids of the eye leading to the loss of central vision. The late stage of AMD with visual loss is classified into two major subtypes, geographic atrophy and exudative AMD. Exudative AMD is characterized by choroidal neovascularization and occurs in 10% of AMD patients, accounting for more than 90% of blindness caused by the disease. Epidemiologic studies show that exudative AMD with no soft indistinct drusens that are characteristics of dry AMD is more prevalent in Asian populations. The etiology of AMD is complex, attributed to multiple genetic and environmental factors. Linkage studies have mapped AMD to 2 chromosomal loci, 1q32 and 10q26. In 2005, a genome-wide association study revealed Y402H-CFH mapped to chromosome 1q32 as an AMD-susceptible variant. But follow-up studies found no association between Y402H-CFH and AMD in Chinese and Japanese populations.

To identify genetic variant(s) that predisposes individuals to the predominant exudative AMD phenotype in the Chinese population, a genome-wide association study was conducted. Positional cloning was then performed on the most prominent gene lying in close proximity of the genetic variant(s) identified to further characterize the critical genetic factors linked to the disease.

In a genome-wide SNP scanning and linkage disequilibrium association study with 96 AMD patients and 130 unrelated control subjects, a new genetic variant, rs10490924, located on chromosome 10q26 was identified. It increased the risk of exudative AMD with an extremely high OR of 11.1 and attributed to the population with large associated PAR of 84%, suggesting a high impact to the disease. rs10490924 lies in a hypothetical gene, LOC387715, with no evidence of biological function. It resides between *PLEKHA1* and *HTRA1*. The frequency of the rare allele at *PLEKHA1* showed no association with AMD. Hence, the other gene, *HTRA1*, was further investigated.

A case-control study involving 163 exudative AMD patients and 183 unrelated control subjects revealed 4 significant SNPs in the promoter and the first exon of *HTRA1*: -491G>A (rs11200638), -359T>C (rs2672598), 230C>T (rs1049331) and 236G>T (rs2293870) with respective p-values = 5.9×10^{-14}, 5.0×10^{-12}, 1.1×10^{-13} and 1.1×10^{-13}. Among them, rs11200638 (-491G>A) is the most significant associated SNP with a very high OR of 7.64 (95%CI: 3.98-14.65). Association of this SNP with eye disease has not been previously reported. Interactive analyses of rs11200638 with smoking and with rs800292 (*CFH*) were conducted. Independent multiplicative effects without significant interaction in either model were found. The

joint disease OR of smoking and rs11200638 (*HTRA1*) caused a 15.71 fold increase in risk whereas that of rs800292 (*CFH*) and rs11200638 showed a 23.3 fold increased risk. An extremely high PAR of 78.4% was also found, indicating a high impact of the additive effect of *CFH* and *HTRA1* in the development of exudative AMD. A new AMD-susceptible *HTRA1*variant was therefore identified in this study as a strong genetic factor underlying the pathogenesis of AMD.

摘要

老年黃斑病變(AMD)是長者常見的眼疾，是導致 60 歲以上長者失明的主要成因。黃斑區位於視網膜中央，當中佈滿感光細胞，其密度更是全視網膜之冠。患上老年黃斑病變人士，中心視力模糊、景物扭曲、顏色減退、影像中央或附近出現盲點。

老年黃斑病變分爲兩大類: 乾性和濕性。乾性是因黃斑點底部的視網膜色素上皮層細胞(RPE)積聚黃白色的沉積物，叫做脈絡膜小疣 (drusen)。使黃斑點失去功能，造成細胞逐步退化，中心視力受損。濕性佔老年黃斑病的一成至一成半，是導致九成老年黃斑病中失明個案的原因。其成因是黃斑區底部出現不正常的新生血管。這些血管滲出液體和血液積聚結疤，破壞視網膜的感光細胞。

目前黃斑病變的原因複雜，研究發現，遺傳、飲食習慣、吸煙、強光照射、心血管病、情緒過度緊張等，都是引致老年黃斑病變的可能原因。連鎖分析研究把老年黃斑病變定位在染色體: 1q32 及 10q26 上。在 2005 年全基因組關聯性研究上，發現 Y402H-CFH 是黃斑病變的危險因子，它的定位是在染色體 1q32 上。但 Y402H 並不是日本及漢族老年黃斑病變患者人群的。

本研究於漢族濕性老年黃斑病變病人及對照組作全基因組關聯性研究(Genome-wide association)，把濕性老年黃斑病變定位在染色體 10q26 的 rs10490924 上，這個危險因子會大大提高致 11.1，而族群相差危險性百分比(PAR)爲 84%。rs10490924 位於 *PLEKHA1* 和 *HTRA1* 之中，尤於 *PLEKHA1* 並沒有發現和老年黃斑病變有關，因此 *HTRA1* 之基因普查研究會幫助我們確定它跟濕性老年黃斑病變的關係。

HTRA1 之病例對照研究中，我們發現 4 個有統計學意義的單核苷酸多態性 (SNP): -491G>A (rs11200638), -359T>C (rs2672598), 230C>T (rs1049331) 和 236G>T (rs2293870)，它們分別的 p-value 爲 $5.9x10^{-14}$, $5.0x10^{-12}$, $1.1x10^{-13}$ 和 $1.1x10^{-13}$。rs11200638 所引致的病例對照 OR 是最大的 (OR=7.64; 95%CI: 3.98-14.65)。相互作用之研究發現吸煙和 rs800292 (*CFH*)跟 rs11200638 (*HTRA1*)皆沒有相互作用，它們和 rs11200638 之關係爲獨立性的乘積性作用。吸煙和 rs11200638 (HTRA1)之聯合疾病 OR 爲 15.71；而 rs800292 (*CFH*) 和 rs11200638 (*HTRA1*) 之聯合疾病 OR 爲 23.3，還發現頗高的族群相差危險性 (PAR)爲 78.4%，顯示出 *CFH* 和 *HTRA1* 對濕性老年黃斑病變的極大累加性作用。

本研究發現 *HTRA1* 的多態性是新的濕性老年黃斑病變之危險因子，值着 *HTRA1* 的功能性研究我們會更瞭解濕性老年黃斑病變之發病原因。

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Abbreviations

Nucleotides

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Amino Acids

Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic Acid
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic Acid
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine
Ter	X	Stop codon

General

ADD	Additive
AMD	Age-related macular degeneration
AP2α	Adaptor-related protein complex 2 alpha

APOE	Apolipoprotein E
APS	Ammonium persulfate
BF	Factor B
bp	Base pair
C2	Complment component 2
CDCV	Common disease-common variant
cDNA	complementary DNA
CFH	Complement Factor H
CI	Confidence interval
CNV	Chroidal neovascularization
CNVM	Choroidal neovascular membrane
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotides
DOM	Dominance
ECM	Extracellular matrix
EDTA	Ethylenediaminetetracetic acid
EtBr	Ethidium Bromide
EtOH	Ethanol
g	Gram
GA	Geographic Atrophy
GCOS	GeneChip Operating software
GDAS	GeneChip DNA Analysis Software
het	Heterozygous
hom	Homozygous
HTRA1	High temperature requirement
HWE	Hardy-Weinberg equilibrium
IGFBP	Insulin-like growth factor domain
INT	Interactive
KI	Kazal-type serine protease inhibitor
kb	Kilobase
L	Liter
LD	Linkage disequilibrium
M	Molar
MAF	Minor allele frequency
μg	Micorgram (10^{-6}g)
μ L	Microliter (10^{-6}L)
mg	Milligram (10^{-3}g)

$MgCl_2$	Magnesium sulfate
ml	Milliliter (10^{-3}L)
mM	Millimolar (10^{-3}M)
μM	Micromolar (10^{-6}M)
mRNA	messenger RNA
MMP	Matrix metalloprotease
OR	Odds ratio
PAR	Population attributable risk
PCR	Polymerase chain reaction
PCV	Polypoidal choroidal vasculopathy
PLEKHA1	Pleckstrin Homology domain containing, family A
RPE	Retinal pigment epithelium
RPED	Retinal pigment epithelium detachment
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SPSS	Statistical Packages for Social Science
SRF	Serum response factor
SNP	Single nucleotide polymorphism
TAE	Tris-acetate/EDTA
TBE	Tris-borate/EDTA
TCF7L2	Transcription factor 7-like 2
TD	Touch down
TEMED	N,N,N' ,N' ,-tetramethyl ethylenediamine
TGF	Transforming growth factor
UTR	Untranslated region
UV	Ultraviolet
χ^2	Chi-square

Publications related to the work of this thesis

1. Chen LJ, Liu DT, **Tam PO**, Chan WM, Liu K, Chong KK, Lam DS, Pang CP. 2006. Association of complement factor H Polymorphisms with exudative age-related macular degeneration. Mol Vis 12:1536-42.

2. Dewan A, Liu M, Hartman S, Zhang SS, Liu DT, Zhao C, **Tam PO**, Chan WM, Lam DS, Snyder M, Barnstable C, Pang CP, Hoh J. 2006. HTRA1 promoter polymorphism in wet age-related macular degeneration. Science 314(5801):989-92.

3. Ng TK, Chen LJ, Liu DTL, **Tam POS**, Chan WM, Liu K, Chiang SWY, Lam DSC, and Pang CP. Association between Complement Factor H Gene Polymorphisms and Exudative Age-related Macular Degeneration in Ethnic Chinese. In preparation.

4. **Tam POS**, Ng TK, Liu DTL, Chan WM, Lam DSC, and Pang CP. Association of HTRA1 and its interaction effect with smoking and CFH variant on risk of exudative age-related macular degeneration. In preparation.

Conference Presentations related to this thesis:

Oral Presentations

1. **Tam POS**. Molecular Techniques in Medical Genetics. The 7th Asia Pacific Society of Eye Genetics-Graduate Research Symposium/Joint Meeting, The 6th International Symposium of Ophthalmology Aug13-15, 2006

Poster presentations

1. **Tam POS**, Chen LJ, Liu TL, Chan WM, Pang CP. Complement Factor H Polymorphisms in Chinese Population with age-related macular degeneration. The 8th International Meeting on Human Genome Variation and Complex Genome Analysis (8th HGV). Hong Kong 14-16 September 2006. Abstract: 101.

2. LJ Chen, DTL Liu, **Pancy OS Tam,** WM Chan, K Liu, KKL Chong, DSC Lam and CP Pang. Ccomplement Factor H Polymorphisms are associated with Exudative Age-related Macular Degeneration. The 6th International Symposium of Ophthalmology, Hong Kong, ASCRS-CUHK Joint Meeting, APVRS Inaugural Symposium, The 7th APSEG Asia Pacific Society of Eye Genetics Symposium, 13-15 August, 2006. Poster # 3 (P-B-3)

Chapter 1

Introduction

1.1. AMD

Age-related macular degeneration (AMD) is a leading cause of severe visual impairment in the United States, Europe and most developed countries, affecting more than 10 million people worldwide (Friedman et al., 2004;la Cour M. et al., 2002). It is a late-onset, chronic and progressive degenerative disorder involving typical lesions in the central region of the retina (macula) and choroid leading to loss of central vision (Figure 1.1). Light enters the eye through the pupil, which is then focused on the back of the eye (retina) by the cornea and the lens located behind the pupil (Figure 1.2). The retina is the light-sensitive nerve layer lining at the back of the eye. Electric impulses are then created and transmitted through the optic nerve to the brain. The macula is the center of the retina, comprising approximately 20% of the total population of cone photoreceptors and a markedly increased density of ganglion cells (Curcio and Allen, 1990). Fovea situated in the center of the macula has the peak concentration of cone photoreceptors. The macula together with the fovea is responsible for fine detail image. AMD, being a progressive disease of the retina, attacks the macula giving rise to central blurred vision or central vision loss (Figure 1.3) but with preservation of peripheral visual field. Its

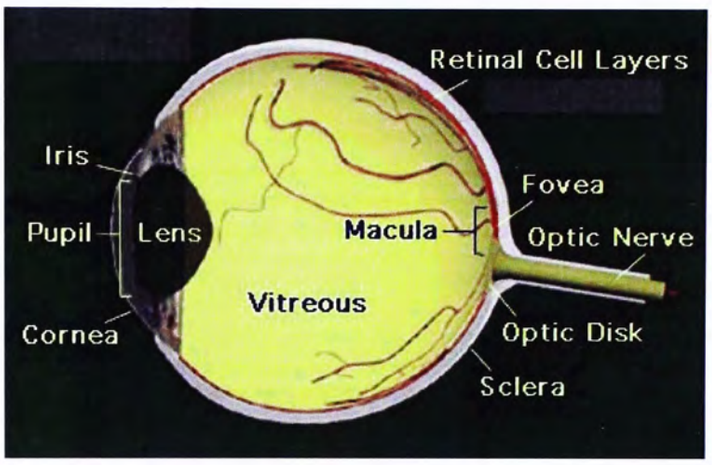


Figure 1.1. Anatomy of the Eye.

The macula is situated in the central region of the retina with fovea approximately in its centre.

(Picture is obtained from online database, MD Support, Inc. & the National Low Vision Support Group.

http://www.mdsupport.org/index.html)

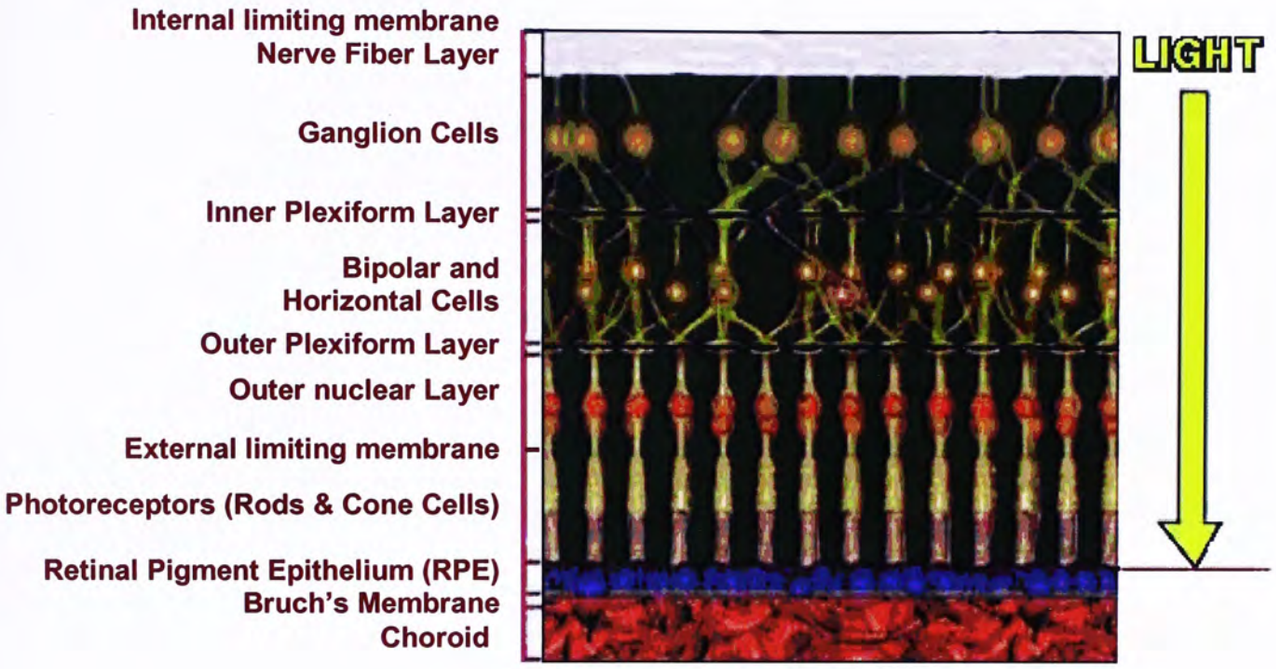


Figure 1.2 Layers of the retina and the choroid

(Picture is obtained and modified from online database, MD Support, Inc. & the National Low Vision Support Group.

http://www.mdsupport.org/anatomy.html)

Figure 1.3 Visual field loss in AMD patients

Left: Wavy and crooked image at the center of vision experienced by patients.

Right: Central vision loss with peripheral image undisturbed.

(Photos are obtained from online database, The Macular Degeneration Partnership. http://www.amd.org/site/PageServer?pagename=Vision_Examples)

clinical and pathological features are alteration of retinal pigment epithelium (RPE) cells and Bruch's membrane, drusen formation, hypo and/or hyperpigmentation, loss of photoreceptors, choroidal neovascularization, and subretinal fibrous and/or neovascular tissue in the macula (Ambati et al., 2003).

1.2. Epidemiology

AMD has a prevalence of 9% of the total population from the group of age 52 to 85 years (Bonastre et al., 2002; Haddad et al, 2006; Klein et al, 2006). It has been reported that about 30% of the population over 75 years of age harbor some degree of AMD (Friedman et al., 2004;Klein et al., 1992;Oneill et al., 2001). Approximately 6-8% of this group will advance to the later stage of the disease, which causes the most severe visual loss. Atrophic AMD and exudative AMD are the two forms of advanced forms of AMD, with atrophic AMD being the most prevalent form (85% of AMD cases). Although exudative AMD is less common, it poses more threats to vision loss. Among these severe cases, nearly 90% are due to the suffer of exudative/wet AMD (Bylsma and Guymer, 2005;Donoso et al., 2006). With the growth of the elderly population, particularly in the developed countries, the incidence of the disease will be expected to increase by 50% (Friedman et al., 2004;Smith et al., 2001). It is of utmost importance to unravel the etiology of AMD so as to prevent or eliminate the threatening of visual impairment and to ease the burden to public health.

1.3. Classification

Early AMD is characterized by the presence of soft drusen with no loss of visual acuity. It is advanced to the later stage of AMD with visual loss and is broadly classified into two clinical forms, namely dry AMD or geographic atrophy and wet or neovascular or exudative AMD. These two clinical forms are further subdivided, according to their specific phenotypic characteristics, to five-step grading systems with geographic atrophy and exudative AMD being the advanced stages (Table 1.1) (Bird et al., 1995;Friedman et al., 2004;Klein et al., 1991;Seddon et al., 2006c). Exudative AMD is known to be the more severe form.

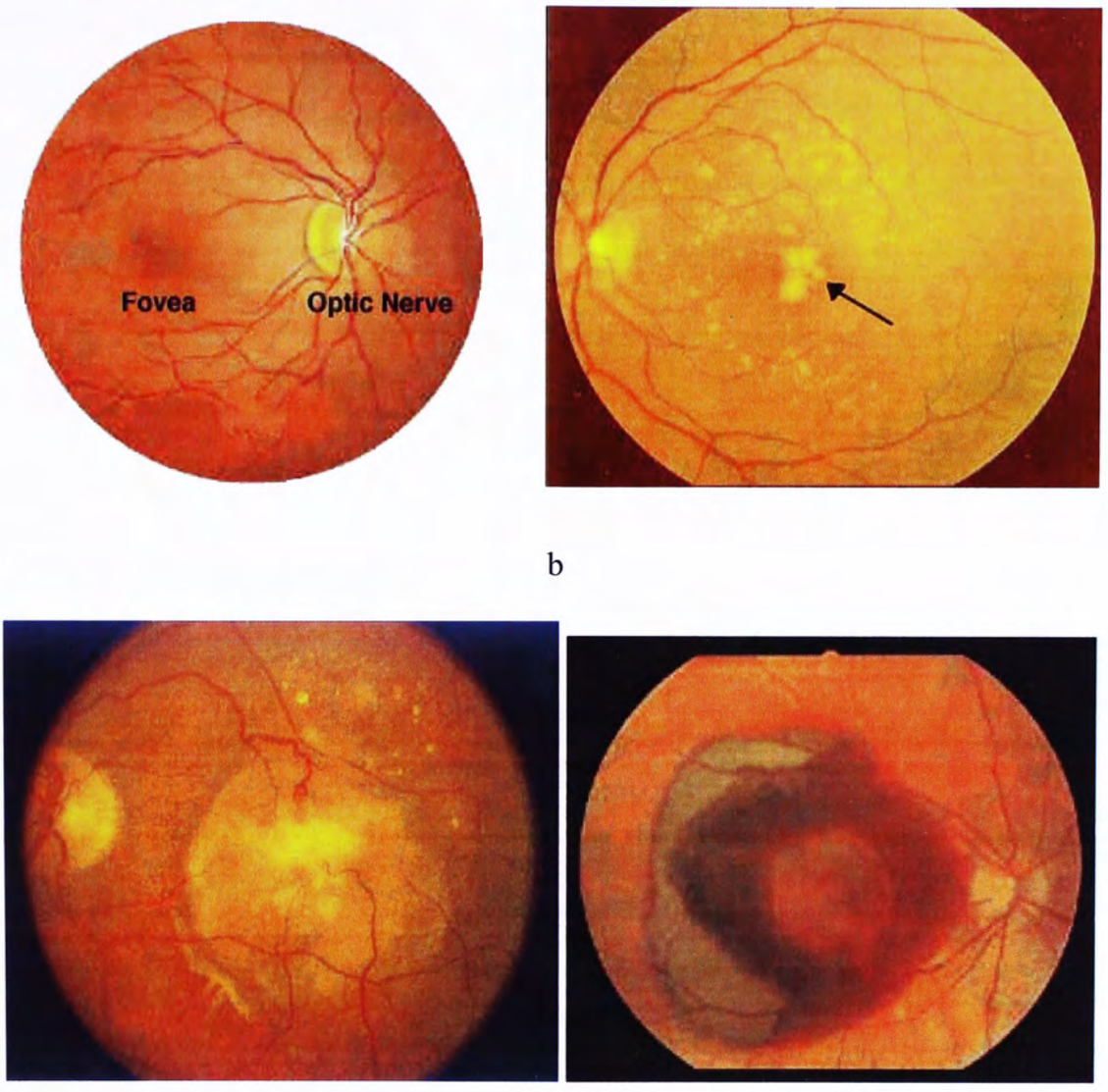
1.3.1. Dry AMD

Dry AMD is the most abundant form of AMD that comprises about 80% of the cases. Drusen are its characteristic feature which are yellow, extracellular deposits of lipid, protein and cellular debris formed subretinally between the Bruch's membrane and the RPE, a layer with photoreceptor cells (Figure 1.4) (Mullins et al., 2000;Russell et al., 2000;Wang et al., 2003). They have been reported to contain complement proteins and inflammatory modulators generated by inflammation (Anderson et al., 2002;Hageman et al., 2001;Hageman et al., 2005;Mullins et al., 2000). Hyperpigmentation or hypopigmentation are observed abnormalities in RPE. Large drusens form from coalescent may lead to RPE detachment. Some of these lesions progress to atrophic area causing geographic atrophy (GA), which is one of the advanced forms of AMD with observed degeneration in the RPE and overlying receptors. Dry AMD can also progress

Table 1.1 The clinical age-related maculopathy (ARM) staging systems (Haddad et al., 2006;Tuo et al., 2004a)

Grade of maculaopathy	Clinical Features
1	No drusen or < 10 small drusen without pigment abnormalities
2	≧10 small drusen or < 15 intermediate drusen, or pigment abnormalities associated with ARM - Drusen - RPE changed (hyperpigmentation and hypopigmentation - Both drusen and RPE changes
3	≧ 15 intermediate drusen or any large drusen - No drusenoid retinal pigment epithelial detachment (RPED) - Drusenoid RPED
4	Geographic atrophy (GA) with involvement of the macular center, or noncentral geographic atrophy at least 350μm in size
5	Exudative AMD, including nondrusenoid pigment epithelial detachments, serous or hemorrhagic retinal detachments, choroidal neovascular membrane (CNVM) with subretinal or sub-RPE hemorrhage or fibrosis, or scars consistent with treatment of AMD - Serous RPED, without CNVM - CNVM or disciform scar

Small = drusen < 63 μm in diameter located within 2 disc diameters (DDs) of the center of the macular; intermediate = drusen ≥63 μm but < 125 μm, located within 2 DDs of the center of the macula; large = drusen ≥125 μm in diameter located within 2 DDs of the center of the macula.



a b

c d

Figure 1.4 Retinal fundus photos

a) Normal retinal

b) Dry AMD with characteristics drusen deposit (arrow)

c and d) Wet/Exudative AMD with choroidal neovascularization

(Picture is obtained and modified from online database, MD Support, Inc. & the National Low Vision Support Group.
http://www.mdsupport.org/anatomy.html)

to wet AMD, a second clinical form. While drusen are a risk factor to GA development, retinal pigmentary irregularities are a significant risk factor for the development of exudative AMD (Bressler et al., 1990). Earlier studies have reported about 90% of the cases to be dry AMD(Ferris, III et al., 1984). Recent studies have revised the prevalence to be about 80% and 20% to be the wet form (Allikmets et al., 1997). To date, no treatment for the atrophic form is available except for the use of antioxidants that delay the progression.

1.3.2. Wet/Exudative AMD

Wet AMD, also known as neovascular AMD or exudative AMD, is another advanced form of AMD that afflicts more severe visual loss than the dry AMD with atrophy. It is characterized by choroidal neovascularization (CNV) and/or RPE detachments. New vessels form under the retina, leading to the destruction of the serous RPE. Blood or fluid leaks from choroidal neovascular membrane, resulting in further scarring. This causes permanent damage to the surrounding retinal tissue, distorting and destroying central vision (Chang et al., 1999;Klein et al., 2006). Epidemiologic studies have shown that wet AMD is more prevalent among Asians than Caucasians, with most of the wet AMD advanced without drusen (Jonasson et al., 2003). Currently, there exists no treatment in AMD that can restore vision of the patient but to slower the progression of the disease. Laser photocoagulation and photodynamic therapy (PDT) are two laser treatments that help to stop further leakage temporarily by coagulating the vessel (Macular Photocoagulation Study Group 1994;Bonastre et al., 2002) . PDT has shown to prevent further visual loss and not to cure (Wormald et al., 2001). Anti-angiogenic drugs

have also been used to treat exudative AMD (Gillies et al., 2003). These treatments are of short-term effect and cannot restore visual impairment of the patients. Anti-vascular endothelial growth factor (anti-VEGF) agents, like ranibizumab and bevacizumab, have been shown to be effective in the treatment of neovascular/exudative AMD but their efficacy and potential risk need further investigation.

1.4. Etiology and risk factors of AMD

With the increase of life expectancy in the human population, the incidence of advanced AMD would be expected to be more prevalent. Identification of the etiology of AMD will be important for the early detection and prevention of the disease. There exists a growing spectrum of known AMD risk factors, whereby environmental, ethnic and genetic factors contribute most to its development, among which age is the most significant risk factor for the development of AMD. Gender, smoking and genetic factor also play a crucial role.

1.4.1. Gender and Ethnicity

Females, who have a longer life span, have a higher risk of advanced AMD (Smith et al., 1997). Besides gender, population-based studies have shown ethnic differences in the prevalence of AMD. Drusen are more prevalent in both blacks and whites over age 40 and elderly whites have more incidence of advanced AMD (Clemons et al., 2005;Friedman et al., 1999;Klein et al., 1995). In epidemiological studies, Asians are found to have a higher incidence of exudative AMD than Caucasians (Bird, 2003;Chang

et al., 1999;Klein et al., 2006;Lim et al., 1998), with the soft drusen found in dry AMD being rarely seen in them (Sandberg et al., 1998;Uyama et al., 2000;Yuzawa, 2000). The prevalence of geographic atrophy is found to be more in Iceland population (4.6% GA prevalence to none in exudative AMD) (Jonasson et al., 2003) and European population (80-85% of AMD cases) (Bonastre et al., 2002), whereas the exudative form of AMD is more prevalent in the Asian population (Chang et al., 1999;Klein et al., 2006;Lim et al., 1998). The observed ethnic prevalence differences can be due to the underlying different genetic background and effects of different risk factors.

1.4.2. Smoking and vascular factors

Consistent population-based association studies have demonstrated that smoking is associated with higher risk of getting advanced AMD (Conley et al., 2006;DeAngelis et al., 2007;Fraser-Bell et al., 2006;Oneill et al., 2001;Seddon et al., 1996;Smith et al., 2001;Vingerling et al., 1996). The population attributable risk for AMD in former and current smokers has reported to be ranged from 20% to 68% (Mitchell P. et al., 1999). Smoking is hypothesized to alter the metabolism of the RPE by depressing the release of antioxidants and alteration of choroidal blood flow. The retina is thus subjected to oxidative stress due to its high consumption of oxygen. The free radicals and extracellular matrix along Bruch's membrane and the increased levels of the angiogenic factors in RPE cells from the insult of oxidative stress will then promote neovascularization (Mousa et al., 1999). Other risk factors like hypertension (Macular Photocoagulation Study Group 1997;Hyman and Neborsky, 2002), alcohol consumption (Fraser-Bell et al., 2006;Mitchell P. et al., 1999;Ritter et al., 1995), estrogen intake and

diet have been reported to show association to AMD risk but findings are being incoherent.

1.4.3. Genetic Factor

Substantial epidemiologic studies suggest that AMD has a significant genetic component involvement. Its complex nature, especially phenotypic heterogeneity, complicates the process of gene discovery. First-degree relatives of advanced AMD patients have shown a higher incidence and a relatively earlier age manifestation in familial aggregation analysis study (Klaver et al., 1998;Seddon et al., 1997). Earlier and recent twin studies reveal concordance of AMD manifestation in monozygotic than in dizygotic twin pairs, which further reinforce the importance of genetic factors in AMD risk (Hammond et al., 2002;Meyers et al., 1995;Seddon et al., 2005).

1.5. Molecular Genetics of AMD

There have been advances in the analytical methods for complex genetic traits. However, the decipher of complex disorder by searching for susceptible loci and/or candidate genes has been less successful than that of simple Mendelian disorders like Huntington's disease and Cystic Fibrosis. There is no evidence for a Mendelian mode of inheritance of AMD, which is believed to be multiple traits with complex etiology. This is also probably due to complicating factors such as intervention of environment factors, incomplete penetrance of the disease, phenotypic and ethnic heterogeneity, polygenic effects and not to mention, the entity of late onset.

1.5.1. Linkage Studies

Genome-wide linkage analysis is widely used over the past decades to map genetic susceptible loci to the disease phenotype. Linkage analyses search for regions in the genome with shared alleles among affected individuals within a family. This indicates that near to the proximity of this linked region there exists a disease-predisposing allele. About 500 polymorphic markers (microsatellite markers) across the genome will be adequate to detect the linked region. This gene mapping approach is powerful to identify rare high-risk disease alleles.

Genome-wide linkage studies of AMD on large pedigrees, affected sib-pairs and discordant sib pairs have mapped a number of susceptible genetic loci 1q, 2q, 3p, 6q, 9q, 10q, 16q 17q and 22q to AMD (Table 1.2) (Abecasis et al., 2004;Iyengar et al., 2004;Kenealy et al., 2004;Majewski et al., 2003;Schick et al., 2003;Schmidt et al., 2004;Seddon et al., 2003;Weeks et al., 2004;Weeks et al., 2001). Among these reported loci, consistent results are repeatedly obtained for 1q31 and 10q26 by different research groups while the other loci have shown to associate with AMD risk with inconsistent findings. A recent genome-scan meta-analysis of six independent studies has further enriched the evidence of linkage on 10q26 and 1q (Fisher et al., 2005). This meta-analysis also shows weak evidence of linkage to chromosome 2p, 3p, 4q, 12q and 16q. Yet no significant disease-causing gene has successfully been identified with subsequent traditional positional cloning method. Following genome-wide scan, candidate genes screening on extended families and sib-pairs study was performed on exon 104 of

Table 1.2 AMD-related chromosomal loci identified by Linkage studies (Klein et al., 1998)

AMD Locus	Marker	Maximum LOD	References
1q25-31	D1S240/D1S412	3.20	(Weeks et al., 2001) (Majewski et al., 2003) (Seddon et al., 2003) (Iyengar et al., 2004) (Weeks et al., 2004) (Seddon et al., 2003)
2q31/2q32	D2S1391/D2S1384	2.32/2.03	(Majewski et al., 2003)
3p13	D3S1300/D3S1763	2.19	(Schick et al., 2003) (Schick et al., 2003)
6q14	D6S1056/D6S249	3.59/3.17	(Majewski et al., 2003)
9q33	D9S934/D9S934	2.06	(Majewski et al., 2003)
10q26	D10S1230	3.06	(Kenealy et al., 2004) (Iyengar et al., 2004) (Weeks et al., 2004) (Jun et al., 2005)
14q32.33	D14S1007		(Schmidt et al., 2004)
16p12	D16S403	2.9	(Weeks et al., 2001)
17q25	D17S928	3.16	(Weeks et al., 2004) (Jun et al., 2005)
19q13.31	D19S245		(Seddon et al., 2003)
22q12	D22S1045	2.0	(Haddad et al., 2006)

Hemicentrin-1 and the 11 exons of *EFEMP1* residing on 1q31 and 2p16, respectively, with no disease causing allele being identified (Iyengar et al., 2004). To date, genome-wide linkage studies have identified associated susceptible loci that help to prioritize subsequent positional and functional candidate genes search. Difficulties to locate the genetic loci for AMD will be: 1) late disease-onset, 2) lack of big pedigrees, 3) variations in phenotype, 4) influences by environmental factors, and 5) heterogeneous and overlapping clinical features.

1.5.2. Candidate genes search

There is not much success in the course of positional cloning after the identification of susceptible loci from linkage studies. Table 1.3 summarizes candidate genes search with negative results (Haddad et al., 2006). In the locus 1q25-31, being mapped from a large US pedigree (Schultz et al., 2003;Schultz et al., 2005;Weeks et al., 2001), one genetic variant, Gln5345Arg, in *HEMICENTIN-1* located on 1q25-31 is identified with segregation in the study family. Yet another group has reported no associated genetic variants with their AMD cases (Schultz et al., 2005;Stone et al., 2004). Table 1.4 summarizes the candidate genes search with at least one positive association (Haddad et al., 2006). Most of the candidate genes searching for AMD have not been successful with mixed findings among different groups of study. The approach of candidate gene search is based on the analysis obtained from previous linkage studies and knowledge of the gene function. Inconsistent findings may be due to the genetic association being related solely to the studied family that might not exhibit the same genetic contribution

Table 1.3 Candidate Gene studies for AMD with negative results to date (modified from Haddad et al., 2006)

Chromosome	Gene	References
1q	ADPRT1, EPHX1, GLRX2, LAMC1, LAMC2, LAMB3, PRELP, RGS19, TGFB2,OCLM	(Conley et al., 2005; Esfandiary et al., 2005; Hayashi et al., 2004)
2p	EFEMP1 (Fibulin 3)	(Guymer et al., 2002; Stone et al., 1999)
2q	IL1A, Fibulin 2	(Haines et al., 2006; Stone et al., 2004)
3p	GPX1	(Esfandiary et al., 2005)
3q	IMPG2	(Kuehn et al., 2001)
6p	RDS	(Shastry and Trese, 1999)
7	AhR	(Esfandiary et al., 2005)
8p	NAT2	(Esfandiary et al., 2005)
10q	CYP2E1	(Esfandiary et al., 2005)
11p	CAT	(Esfandiary et al., 2005)
11q	Fibulin 4, VMD2	(Akimoto et al., 2001; Allikmets et al., 1999; Kramer et al., 2000; Seddon et al., 2001; Stone et al., 2004)
12p	A2M, MGST1	(Haines et al., 2006)
14q	CKB	(Haines et al., 2006)
15q	CYP1A1, CYP1A2	(Esfandiary et al., 2005)
17q	APOH, ITGB4	(Conley et al., 2005)
22q	CYP2D6, Fibulin 1, TIMP3	(De La Paz et al., 1997; Esfandiary et al., 2005; Stone et al., 2004)

Table 1.4 Candidate Gene studies for AMD with at least one positive findings to date (modified from Edwards et al., 2005)

Chromosome	Gene	Reference
1p	ABCA4	(Allikmets, 2000; Allikmets et al., 1997; Baum et al., 2003; Bernstein et al., 2002; Fuse et al., 2000; Guymer et al., 2001; Kuroiwa et al., 1999; Rivera et al., 2000; Schmidt et al., 2003; Shroyer et al., 1999; Souied et al., 2000; Webster et al., 2001)
1q	HEMICENTIN (Fibulin 6)	(Abecasis et al., 2004; Conley et al., 2005; Hayashi et al., 2004; Iyengar et al., 2004; Schultz et al., 2003; Stone et al., 2004)
3p	CX3CR1	(Tuo et al., 2004b)
6p	HLA Genes, VEGF	(Churchill et al., 2006; Goverdhan et al., 2005; Haines et al., 2006)
6q	ELOVL4, SOD2	(Ayyagari et al., 2001; Conley et al., 2006; Esfandiary et al., 2005; Kimura et al., 2000)
7q	PON1	(Baird et al., 2004; Esfandiary et al., 2005; Ikeda et al., 2001)
9p	VLDLR	(Conley et al., 2005; Haines et al., 2006)
9q	TLR4	(Zareparsi et al., 2005)
12p	LRP6	(Haines et al., 2006)
14q	Fibulin 5	(Stone et al., 2004)
17q	ACE	(Conley et al., 2005; Haines et al., 2006; Hamdi et al., 2002; Hamdi and Kenney, 2003)
19q	APOE	(Baird et al., 2006; Conley et al., 2005; Gotoh et al., 2004; Pang et al., 2000; Schmidt et al., 2000; Simonelli et al., 2001; Souied et al., 1998; Zareparsi et al., 2004)
20p	CST3	(Zurdel et al., 2002)
20q	MMP9	(Fiotti et al., 2005)

as in other studied families. Also, these observed genetic variants usually have mild genetic influence to the disease.

Many investigators have proposed that inherited macular dystrophies, such as Best's, Stargadt's, Sorby's fundus, and peripherin/RDS related dystrophy, could be potential models for AMD. Various genes from some of these inherited macular diseases are chosen to perform further analysis on AMD patients since these monogenic macular and retinal diseases share similar clinical and pathological features with AMD. Contribution of these variants to overall AMD prevalence and development appears relatively insignificant since independent replication of the findings has been unsuccessful.

Recently, follow-up on the identification of the AMD-susceptible locus on 10q26, subsequent analysis of the region by 93 SNPs for allelic association was performed by a group of researchers. With this different study approach, a hypothetical gene, LOC387715, as a susceptible gene to AMD risk was identified (Rivera et al., 2005).

Recent findings from genome-wide association study have demonstrated Complement Factor H (*CFH*) conferring significant AMD risk, which will further be discussed in later section. Candidate gene approach given by the pre-knowledge of the complement system has led to the study of other genes in the system, factor B (*BF*) and complement component 2 (*C2)*. These two genes are located in chromosome 6p and have been found to carry risk haplotypes and protective haplotypes to AMD (Gold et al., 2006).

1.5.3. Genome-Wide Association

Linkage is known to map disease locations/loci. Association is to match the co-occurrence of specific allele(s), genotype, haplotype at a specific locus/loci and the disease trait. The advent of HapMap project has identified many patterns of human genetic variation across the genome. This genetic variation, being the single nucleotide polymorphisms (SNPs), occurs ubiquitously across the genome in coding, non-coding and untranslated region. SNPs being much more common in the genome provide a higher resolution in genetic mapping. The common disease-common variant (CDCV) hypothesis predicts that common disease causing alleles will be found in populations. Complex polygenic diseases, which involve multiple genetic and environmental factors, are evolutionary neutral and are caused by these variations (Pritchard and Cox, 2002). Each variation in a complex disease will contribute a small effect on the disease phenotype resulting in additive or multiplicative effect of many susceptible alleles (Carlson et al., 2004;Collins et al., 1997;Yang et al., 2005). Susceptible variants involved in complex disease usually have low to medium penetrance. While linkage analysis approach is powerful to identify rare high-risk disease alleles, association analysis approach has better power to detect common disease arising from modest disease risk. The identification of inconsistent disease loci from linkage studies demonstrates its insufficient detection power and the difficulty in the study of the common disease with common variant hypothesis. Disease-predisposing variants are suggested to exist in the population at relatively high frequency. In order to identify genetic variants underlying complex disorders, very dense SNPs maps, providing adequate coverage of the human genome, must be used (Cardon and Palmer, 2003;North

et al., 2004). With the advancement of high throughput genotyping technique, this genome-wide association study approach has become more affordable.

Association analysis is the speculation of the allele frequency of a set of polymorphic markers between cases and unrelated controls, with higher frequency in cases conferring a disease susceptible allele or a marker allele in linkage disequilibrium with the disease-causing allele.

1.5.3.1. Complement Factor H

Recently, genome-wide association approach has identified a non-synonymous SNP encoding Y402H in complement factor H gene (*CFH*) on chromosome 1q32 being significantly associated to the risk of dry AMD (Klein et al., 2005). This genetic variation is then replicated in other studies with similar significance level (Edwards et al., 2005;Hageman et al., 2005;Haines et al., 2005). Consistent replications are also found in other ethnic population, French (OR_{het}=3.00; OR_{hom}=6.93 to exudative AMD) (Souied et al., 2005), Dutch (OR=11.02 to Stage 4 AMD) (Despriet et al., 2006), Italy (OR=3.9) (Simonelli et al., 2006), Russian (OR=2.71) (Fisher et al., 2006), Indian (OR =11.52; p-value=1.19×10^{-7}) (Kaur et al., 2006), German (OR_{hom}=6.72) (Rivera et al., 2005) and Taiwan-Chinese (OR=4.4; p-value=10^{-6}) (Lau et al., 2006). Table 1.5 summaries the findings on *CFH* Y402H prevalence on different ethnic populations. Divergent frequencies of *CFH* Y402H variant exist among different ethnic populations. In the Japanese population, a lower disease allele frequency of Y402H (MAF=0.04) than in the Caucasians (MAF=0.34) is observed that confer no association to exudative AMD (Fuse

Table 1.5 Odds ratio of *CFH* Y402H polymorphism conferring AMD in different ethnic populations

Ethnic Population	AMD	OR_{hom} (Y402H)	References
Americans	395 (Dry or neovascular AMD)	4.54	(Klein et al., 2005)
Americans	95 (Dry or neovascular AMD)	8.50	(Souied et al., 2005)
French	141 (Neovascular AMD)	6.93	(Despriet et al., 2006)
Netherlands	171 (GA and CNV)	11.02	(Simonelli et al., 2006)
Italian	104	3.9	(Seitsonen et al., 2006)
Finnish	154 (Dry and Exudative AMD)	9.33	(Rivera et al., 2005)
German	1166 (GA and CNV)	6.72	(Fisher et al., 2006)
Russian	151 (GA and CNV)	2.71	(Magnusson et al., 2006)
Icelandic	581 (GA, Neovascular and Mixed AMD)	2.39	(Kaur et al., 2006)
India	100 (Dry, GA and neovascular AMD)	11.52	(Lau et al., 2006)
Taiwan-Chinese	163 (Neovascular AMD)	4.4	(Gotoh et al., 2006)
Japanese	146 (Exudative AMD) 80 (Dry AMD)	(MAF : 0.04)	(Uka et al., 2006) (Fuse et al., 2006) (Matsuzaki et al., 2004)
Chinese	163 (Exudative AMD)	(MAF : 0.04)	(Chen et al., 2006)

Abbreviations: OR_{hom}, Odds Ratio of homozygous C allele
GA, Geographic atrophy
CNV, Choroidal neovascular AMD
MAF, Minor allele frequency

et al., 2006;Gotoh et al., 2006;Uka et al., 2006). Low minor allele frequency (MAF=0.04) of Y402H is also observed in the Chinese population which harbors no association to exudative AMD subjects (Chen et al., 2006). In addition, they have also found other susceptible alleles in the promoter and coding regions of *CFH* and in different LD blocks to Y402H being associated to their exudative Chinese AMD subjects (Chen et al., 2006). It has long been known that AMD prevalence varies differently in different ethnicities (Evans, 2001;Hsu et al., 2004;Klein et al., 2006;Oshima et al., 2001;Varma et al., 2004) and so is phenotypic heterogeneity. This ethnic variation in AMD-associated *CFH* polymorphism suggests the possibility of other unidentified genetic factors important to the pathogenesis of AMD. These factors may act independently or to moderate the effects of the Y402H variant (Grassi et al., 2006).

CFH is a regulator of the alternative pathway in the complement system. The complement system involves in the immune defense mechanism. The SNP at Y402H conferring risk to AMD is shown to have significant abnormal expression in the drusen (Mandal and Ayyagari, 2006). This genotype-driven abnormal regulation of the complement cascade within Bruch's membrane and adjacent retinal pigment epithelial cells result in uncontrolled complement activation and consequent drusen formation (Sivaprasad and Chong, 2006).

1.5.3.2. LOC387715

Association approach has also been used as a gene discovery screening tool to fine map on the susceptible disease loci being found in linkage studies. Numerous studies have

reported *LOC387715* as a second major locus related to the pathogenesis of AMD. *LOC387715* is a hypothetical gene residing on 10q26. A non-synonymous coding SNP, A69S, in *LOC387715* confers a significant association to AMD risk with 7.6-fold increased risk in homozygote carrier (Rivera et al., 2005). These findings are replicated in an independent case control cohort (Francis et al., 2007). *CFH* Y402H variants also exhibit strong association to this study cohort. These results suggest an independent contribution effect of *CFH* and *LOC387715* on AMD risk. Other groups also replicate the same findings with *LOC387715* conferring significant risk to AMD (Conley et al., 2006;Jakobsdottir et al., 2005;Schmidt et al., 2006;Shastry, 2006).

1.6. Statistical Analysis

1.6.1. Genotyping

Microarrays have been widely used for gene expression study and genomic variation study. For genomic variation study, Comparative Genome Hybridization (CGH) array and SNP-based array are the two common types. SNPs occur one in every 300 to 1000 base pairs throughout the genome. In genomic research, SNP microarrays are used in the attempt to identify DNA sequence variants in specific genes or regions of the human genome that are responsible for a variety of phenotypic traits, such as disease risk or variable drug response. The Affymetrix genotyping platforms provide thousands of SNPs from the human genome on a single chip. With the advances in photolithography-based array technology, feature sizes are reduced from 18μm down to 8μm, which

increase the density five-fold to ~2.5 million unique probe sequences per array. The GeneChip® Human Mapping 100K array (Affymetrix Inc, Santa Clara, California, USA) is available on two chips. Recently, the 100K SNP set has been successfully utilized in the search of a highly significant susceptible disease allele, *CFH* Y402H, to the risk of dry AMD with the presence of large drusen (Klein et al., 2005). The genome coverage of the 100,000 SNPs is shown in Table 1.6. The highly dense genome coverage of this SNP chip will be of high opportunity to identify genomic variants associated with the disease studied.

The SNP arrays contain probe sets to interrogate the two alleles for all the SNPs. 25-mer oligonucleotide probes corresponding to a perfect match for the allele sequence and to a perfect match for the other allele sequence. In addition, a mismatch probe is synthesized for each allele to detect non-specific binding. This quartet probe is the basic unit for detecting different genotype groups: AA, AB or BB. A new dynamic model-based algorithm DM for the Mapping 100K array is used to call the genotype.

1.6.2. Quality Assessment of Genetic Data

Analysis of genotyping data created from association study is complex. Firstly, Hardy-Weinberg equilibrium must be tested for each control genotype. Its violation indicates existence of population stratification in the study, systematic errors in genotyping, random chance, or presence of homologous region in the genome (Cardon and Palmer, 2003). Those genotyping data should be critically examined to scrutinize any genotyping errors and the impact from HWE violations (Trikalinos et al., 2006). Markers with violation of Hardy-Weinberg equilibrium should be excluded in the subsequent analysis.

Table 1.6 Genome coverage Affymetrix GeneChip® Human Mapping array (Nicolae et al., 2006)

Genotyping over 100,000 SNPs
Genome coverage

Range	All SNPs	Hind SNPs	Xba SNPs
± 2000 bp	11.4%	5.8%	6.0%
± 5000 bp	24.4%	13.3%	13.7%
± 10000 bp	40.5%	23.9%	24.6%
± 20000 bp	59.9%	39.9%	41.1%
± 50000 bp	81.9%	66.2%	67.5%
± 100000 bp	91.6%	82.7%	83.9%
± 500000 bp	98.8%	98.0%	98.2%
± 1000000 bp	99.1%	98.9%	99.1%
± 5000000 bp	99.1%	99.1%	99.1%

Genome Coverage:

Total genome size was 3.069 Gbases of which Gaps (centromeres, telomeres, and heterochromatin) accounted for 0.226 Gbases, leaving an effective genome size of 2.843 Gbases. Chromosome size and Gap information for genome Build 34 was downloaded from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgText). For each chromosome, non-overlapping ranges about each SNP site were summed, and then divided by the effective size of the chromosome to estimate genome coverage. Chr_random chromosomes were omitted.

1.6.3. Association Analysis

Each marker is analyzed individually for association to the disease. Chi-square test with one degree of freedom is performed to test for association. If expected cell counts are small (<5), Fisher's exact test is preferred (Clayton and McKeigue, 2001). Odds ratio (OR) is widely used as a relative measure of association. The OR for disease is the ratio of allele carriers to non-carriers in cases compared with that in controls. It indicates the increase in disease risk for carriers comparing to non-carriers. In the study, the less frequent allele in the control group was considered to be the risk allele.

1.6.4. Population Stratification

Population structure is a factor to be addressed for spurious association observed. Population stratification refers to differences in allele frequencies between cases and controls due to systemic differences in ancestry rather than association of genes with disease. This difference will contribute to spurious association. This confounding of subpopulations will need to be tested and corrected before performing any further association analysis. Genomic control is one of the methods for this correction. It treats the population ancestry as a nuisance parameter within the modeling, and removes the effect from the association test statistic for correction (Devlin and Roeder, 1999).

1.6.5. Haplotype Analysis of Multiple SNPs

Substantial evidence has accumulated that the genome can be divided into haplotype blocks of variable lengths (Daly et al., 2001;Gabriel et al., 2002). Within these blocks,

LD is strong, that is, the alleles at different loci are not randomly assorted but allelic states are highly correlated. While between blocks, LD is disrupted, presumably due to historical recombination. Closely linked alleles within the haplotype block tend to be inherited together. If a haplotype contains the disease allele, the subsequent recombination events will carry the disease allele along. Haplotype analysis will help to determine if there exists an increase risk of developing disease. It is mentioned to provide a greater power to detect association than single SNP analysis (Clayton and McKeigue, 2001;Johnson et al., 2001).

Haploview (Barrett et al., 2005) is designed as a tool to analyze, interpret, visualize and depict haplotype block structure using accelerated E-M algorithm. Haplotype block can help to identify closely linked alleles that are in linkage disequilibrium, which can then be employed to identify genetic regions associated with disease. Gold is another software that provides a graphical summary of linkage disequilibrium in human genetic data (Abecasis and Cookson, 2000).

1.6.6. Population Attributable Risk

Population attributable risk (PAR) estimates the proportion of disease in the study population that is attributable to the exposure. A general formula for the calculation of the PAR is (Bruzzi et al., 1985):

$$PAR = P_r(OR - 1)/ (1 + P_r (OR- 1))$$

, where P_r is the prevalence of the risk factor in the general population.

This is the measure of the effects of exposures.

1.6.7. Interaction Analysis

Most common diseases arise from interaction between multiple genetic variations and factors such as environment. The interaction between genetic polymorphisms is referred to as epistasis, which means that the effect of one locus varies according to the genotype present at another locus. It has been stated that epistasis accounts for a situation where a 'masking' gene prevented another phenotypic gene from exhibiting its effects (Cordell, 2002). Logistic regression with modeling of joint loci effect is one approach to study interaction effect of two loci on disease phenotype (Cordell, 2002). Models are fitted into the additive effect and dominance effect of each disease locus and the between-loci additive and dominance interactions. The effects of four alleles present at two loci are partitioned into main effects and various interaction terms. The multiplicative model represents epistasis (Risch, 1990). Epidemiologically, AMD is a complex disorder with contributions of both environmental and genetic risk factors. Smoking is known as one of the most important risk factors of AMD (Seddon et al., 2006a; Seddon et al., 2006b). Recent association study has identified *CFH* as a major AMD-predisposing gene (Klein et al., 2005). The investigation of gene-environment and gene-gene interaction will help to further understand the pathogenesis of AMD.

1.7. Objectives

Linkage studies have repeatedly mapped AMD to two chromosomal loci, 1q32 and 10q26. The Y402H variant of the CFH gene on chromosome 1q32 has been shown to be a major risk factor to dry type of AMD, especially with presence of large drusen. We have recently shown that sequence variants of *CFH*, including rs3753394 (p=0.003), rs800292 (p=0.00053), and rs1329428 (0.00092), but not rs1061170 (Y402H), have significant associations with exudative AMD in the Chinese population. Our data have shown both ethnic constitutional differences and probable inductive differences in the molecular genetics of AMD that may affect their phenotypic expressions, and consequently cast important influences on the modes of treatment. In the work described in this thesis, we confined to Chinese exudative AMD patients and focus on the putative AMD locus on chromosome 10q26:

1. to search for the associated gene of AMD,
2. to identify the gene variants that cause or predispose to development of AMD, and
3. to examine gene-environment and gene-gene interaction effects.

We utilized genome-wide linkage disequilibrium association, positional cloning and direct sequencing in this study.

Chapter 2

Materials and Method

2.1. Materials

2.1.1. Proteins

All restriction endonucleases and T4 DNA Ligase were purchased from New England Biolabs, MA, USA. ExoSap-IT was obtained from GE HealthCare Bio-Sciences, Little Chalfont, United Kingdom. Proteinase K was purchased from QIAGEN Gmb, Hilden, Germany. Platinum Taq polymerase, Platinum Pfx DNA polymerase and Human Cot-1 were obtained from Invitrogen Corporation, Carlsbad, CA, USA. Anti-streptavidin (goat) biotinylated antibody was purchased from Vector Laboratories, USA.

2.1.2. Chemicals

Gene Page Plus 5.25% 6M urea and N,N,N',N'-tetramethyl ethylenediamine (TEMED) was purchased from AMERESCO, Solon, OH, USA. Agarose, ammonium acetate, ammonium persulfate (APS), β-mercaptoethanol, boric acid, bromophenol blue, denhardt's solution, dextran, dimethylsulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA) di-sodium salt dihydrate, ethanol (absolute), ethidium bromide, glycerol, isopropanol, methanol, potassium chloride, potassium dihydrogen phosphate, sodium

bicarbonate, sodium chloride, sodium dihydrogen phosphate, tris base , Tween-20 and xylene cyanol FF were obtained from Sigma-Aldrich Co., St. Louis, MO, USA. 50X TAE and 10x TBE were purchased from Bio-Rad Laboratories, Hercules, CA, USA. dNTP set was obtained from Roche Diagnostics GmbH, Penzberg, Germany. Adaptor Xba and adaptor Hind were product of Affymetrix Inc, Santa Clara, USA.

2.1.3. Solutions and Buffers

Loading Buffer, 6X	0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol
RLT buffer, working stock	RLT buffer with 10µl/ml β-mercaptoethanol
TAE buffer, 50X	2M Tris-Acetate, 50mM EDTA, pH8.3
TBE buffer, 10X	1.0M Tris, 0.9M Boric acid, 10mM EDTA, pH8.4
SSPE buffer, 20X	3M sodium chloride, 0.2M sodium dihydrogen phosphate, 0.02M EDTA

2.1.4. Reagents and kits

Affymetrix GeneChip® Human Mapping 100K Set of microarrays and GeneChip Mapping Assay were purchased from Affymetrix Inc, Santa Clara, USA. QIAamp DNA Blood Midi kit was obtained from Qiagen, Hilden, Germany. BigDye Terminator Cycle Sequencing Reaction Kit v3.1 was purchased from Applied Biosystems, Foster City, CA, USA. Fifty base pair and 1 kilo base DNA ladder and custom primers for sequencing and PCR were obtained from Invitrogen Corporation, Carlsbad, CA, USA.

2.1.5. Apparatus

GeneChip Scanner 3000 and Fluidics Station were obtained from Affymetrix Inc, Santa Clara, USA. ABI 3130XL automated DNA sequencer and GeneAmp PCR System 9700 were purchased from Applied Biosystems, Foster City, CA, USA. All plastic wares were obtained from Labcon Inc., CA, USA. Gel-Doc 2000 video gel documentation system was purchased from Bio-Rad Laboratories, Hercules, CA, USA. Nanodrop ND-1000 was purchased from NanoDrop Technologies, Rockland, DE, USA. Mupid-2 Mini-Gel Electrophoresis System was obtained from Cosmo Bio Co. Ltd., Tokyo, Japan.

2.1.6. Softwares

GeneChip Operating software (GCOS) and GeneChip DNA Analysis Software (GDAS) was obtained from Affymetrix Inc, Santa Clara, USA. ABI Prism 3130XL Sequence Collection and SeqScape were purchased from Applied Biosystems, Foster City, CA, USA. Quantity One® Version 4.0.3 Image Analysis System was obtained from Bio-Rad Laboratories, Hercules, CA, USA. Statistical Packages for Social Science (SPSS) version 11.3 was purchased from SPSS, Chicago, IL. R language and environment for statistical computing version 1.4.0 was downloaded from The R project for Statistical Computing Homepage (http://www.R-project.org).

2.2. Methods

2.2.1. Study Subjects

The study protocol was approved by the Ethnics Committee for Human Research of the Chinese University of Hong Kong. All the procedures used in the study conformed to the tenets of the Declaration of Helsinki. Informed consent was obtained from all study subjects after explanation of the nature and possible consequences of the study. Detailed family and medical histories were documented. Peripheral venous blood was taken from each subject for genetic studies.

2.2.2. AMD Patients

All participants received a standard examination protocol and visual-acuity measurement. All subjects underwent a detailed eye examination, including best corrected visual acuity and slit lamp biomicroscopy of the fundi. Stereoscopic color fundus photographs were taken using the standard classification suggested by the International Age-related Maculopathy Epidemiological Study Group. Patients with exudative AMD had nondrusenoid RPED (retinal pigment epithelium detachment), CNV (chroidal neovascularization), serous or hemorrhagic retinal detachments, subretinal or sub-RPE hemorrhage or fibrosis. In addition, smoking habits were recorded. A smoker is defined as a person who smoked at least 5 cigarettes daily for more than 1 year. Smokers were divided into two groups: those who had never smoked, and those who were ex-smokers and current smokers. A total of 164 exudative AMD were recruited with 88 males and 75 females. The age at diagnosis ranged from 60-94 years, mean 75.5 ± 7.5 years. For the genome-wide association study, those cases over 90 years of age were excluded to closely match the age distribution between cases and controls. A final of 96

cases were used in the study. Follow-up on the candidate gene screening, all of the 164 exudative AMD were analyzed in the study.

2.2.3. Control Subjects

The control subjects did not have family history of AMD or showed no sign of AMD or any other major eye diseases except senile cataracts. Fundus examination was normal for the control subjects. A total of 183 controls were recruited and used in the candidate gene screening study, among which 130 were used in the genome-wide association study since those control subjects younger than 65 years of age were excluded. The characteristics of the final group of 96 cases and 130 controls are given in Table 2.1.

2.2.4. DNA Extraction and Quantification

Genomic DNA was extracted from EDTA-blood using Qiagen QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's Blood and Body Fluid Spin Protocol. The final genomic DNA was eluted with 400μl sterile milli-q water. The extracted DNA was quantified by UV-Visible spectrophotometer, Nanodrop ND-1000 (NanoDrop Technologies, Rockland, DE, USA).

2.2.5. Whole genome wide SNP scanning

Each cases and control subjects were genotyped using the Affymetrix GeneChip® Human Mapping 100K Set of microarrays (Affymetrix Inc, Santa Clara, USA). The SNP genotyping assay consisted of two chips (XbaI and HindIII) with 58,960 and 57,244 SNPs, respectively. Approximately 250ng of genomic DNA was digested with two

Table 2.1 Characteristics of cases and controls in the genome-wide association study

	Exudative AMD	Controls
Total	96	130
Males (%)	68	33
Mean Age (±s.d.) (years)	74.9 ± 6.8	74.2 ± 5.7
Age range (years)	69 - 89	65 – 99
Smokers	63	26

restriction enzymes XbaI and HindIII (New England BioLabs Inc, MA, USA), followed by GeneChip Mapping Assay (Affymetrix Inc, Santa Clara, USA) according to the manufacturer's protocol. Figure 2.1 shows the flowchart of the procedure. The images were scanned on GeneChip Scanner 3000 (Affymetrix Inc, Santa Clara, USA) and analyzed using GeneChip DNA Analysis Software (GDAS) (Affymetrix Inc, Santa Clara, USA). For each chip, two internal control measures were used: the call rate always exceeded 90% and heterozygosity on the X chromosome correctly identified the gender of the individual. To ensure that no samples were confused, identical SNPS placed on both chips were checked for yielding the same genotype for the same individual.

Three experiments were done to assure reproducibility of the system. First, four samples were processed twice with the Xba chips. Next, two replicates of a reference DNA positive control provided by Affymetrix were run on Xba chips alongside the samples described here. Finally, results for three individuals were compared with genotyping using the Affymetrix 10K SNP platform for accuracy test. These hybridization experiments were performed in Dr J Hoh's laboratory of Yale University.

Those individual chips achieving a call rate of >90% were used for the analysis. One Xbal chip was eliminated due to an extremely low call rate (76.72%). This sample was not genotyped on the HindIII chip. One individual was not successfully genotyped on either chip, and three others only when using the HindIII chip. This left with 268

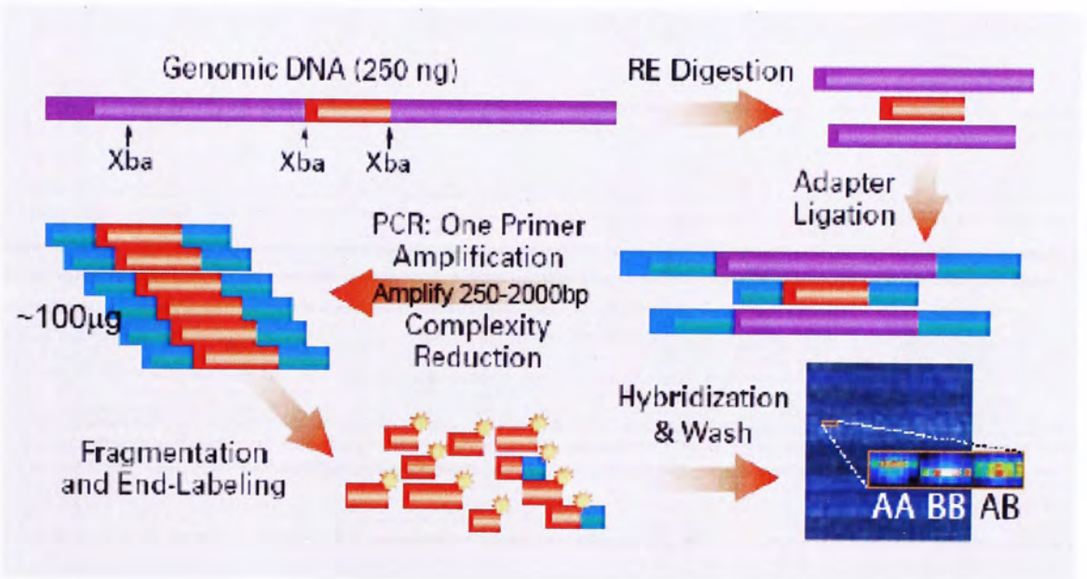


Figure 2.1 Flow chart of the Genotyping procedure of the Affymetrix GeneChip® Human Mapping set (www.affymetrix.com)

individuals genotyped for HindIII and 266 for XbaI (266 of which were genotyped for both chips).

After eliminating this one chip, individual autosomal SNP data quality was assessed by examining the call rates. SNPs with call rate <85% were eliminated from the analysis. To further eliminate SNP with possible genotyping errors, heterozygous SNPs without observed heterozygotes and SNPs with only heterozygotes were excluded. To eliminate uninformative SNPs, non-heterozygous SNPs were excluded. Finally, deviations from Hardy-Weinberg equilibrium (HWE) were assessed, and SNPs with a HWE χ^2>50 were excluded. These exclusions were largely due to low call rates of <85%; hence, 97,824 autosomal SNPs were left for analysis. These data are summarized in Table 2.2.

2.2.6. *HTRA1* Genotyping

With the susceptible SNP to risk of exudative AMD identified as rs10490924, the *HTRA1* gene, lying next to it and in LD with it was chosen for candidate gene screening. The cases and controls were selected with the same criteria as in the genome-wide association study. A final of 163 exudative AMD and 183 control subjects were chosen for the analysis.

2.2.6.1. Serial Polymerase Chain Reaction

PCR reactions were carried out in a total volume of 25 µl containing 20 mM Tris-HCl (pH8.4), 50 mM potassium chloride (KCl), 1.5mM magnesium chloride ($MgCl_2$), 0.5 µM of each amplicon specific primer, 0.2 µM deoxynucleotide triphosphate (dNTP) mix,

Table 2.2 Genotyping data quality

Number of Individuals	
Hind	268
Xba	267
Pre-chip data quality	
Median call rate per chip (Hind)	99.41%
Median call rate per chip (Xba)	99.33%
Minimum call rate per chip (Hind)	94.33%
Minimum call rate per chip (Xba)	76.72%*
Per-individual data quality	
Average number of matches for common SNP between two chips#	30.7
Minimum number of matches for common SNP between two chips#	26
Total Number of SNPs	116204
Number of Autosomal SNPs	113841
Call rate (per SNP)	
SNPs with 100% call rate	71156
SNPs with call rate between 85% and 100%	41934
SNPs with call rate less than 85%	751
SNPs with call rate above 85% (Hind; 40 or less No Calls)	113090
Locus Polymorphism (for autosomal SNPs with call rates > 85%)	
Number of SNPs with no polymorphism observed	14867
Number of SNPs with only heterozygotes observed	17
Number of polymorphic SNPs with no heterozygotes observed	36
Number of SNPs with minor allele frequency <0.01	6008
Hardy-Weinberg Equilibrium (for polymorphic SNPs regardless of MAF)	
Number of SNPs with HWE $\chi^2 > 50$	346
Final number of SNPs	**97824**

* After the one Xba chip with low call rate was removed, there were 266 samples genotyped on the Xba chip and the minimum call rate was 95.85%.

Out of the 31 SNPs that are in common between the two chips.

1 unit of recombinant Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) and 200ng template DNA. The PCR temperature profile consisted of denaturation of the DNA at 94°C for 5 minutes, cycling 40 cycles at 94°C for 40 seconds, an amplicon specific annealing temperature for 50 seconds and 72°C for 40 seconds. This was followed by a 7-minute extension step at 72°C. All primer sequences and amplicon specific annealing temperature are listed in Table 2.3.

2.2.6.2. Cycle Sequencing

10µl of PCR products were treated with 2µl ExoSap-IT (GE HealthCare, USA) and incubated at 37°C for 30 minutes to remove excessive primers and dephosphorylate excessive dNTPs before cycle sequencing. The enzymes were then inactivated at 72°C for 15 minutes. The purified DNA products were subjected to cycle sequencing using BigDye Terminator Cycle Sequencing Reaction Kit (v3.1, Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol on ABI 3130XL capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing data were aligned by SeqScape (Applied Biosystems, Foster City, CA, USA) and analyzed accordingly. The data were compared with the reference sequence, ENSG00000166033, from Ensembl webpage.

2.3. Statistical Analysis

2.3.1. Hardy-Weinberg Equilibrium Test

Table 2.3 *HTRA1* primer sequences and PCR conditions

Amplicon	Forward Primer	Reverse Primer	PCR Conditions Annealing temp °C $MgCl_2$, 10% DMSO	PCR Product (bp)
Promoter	CGGATGCACCAAAGATTCTCC	TTCGCGTCCTTCAAACTAATGG	62-55 (TD) 2.5 mM, DMSO	568
Exon 1	GTCCCCAAGGCGGCTCGT	ACACCTCGCAGCAGCCGC	62-55 (TD) 1.0 mM, DMSO	819
Exon 2	ACGTTTTTGTGGTGAACCTGAGC	GCAACAGCCACACACACCTAGC	62-55 (TD) 1.0 mM	688
Exon 3	GCCCGATATATAAAGGAGCGATGG	AGAAGTTTTCCTGAGCCCCTTCC	62-55 (TD) 1.0 mM	522
Exon 4	GGGATGTTAGTTGTGAGCTCAGTTCC	GCACTAGCCTCCACATGGCTTGG	62-55 (TD) 1.0 mM	485
Exon 5	CTGGGCTTCAGAGAGAAAATCTCC	ATCCGTAGGGTCATTTGCAAGC	62-55 (TD) 1.0 mM	451
Exon 6	AGTGCCGACCTGGAGTATGTGC	GGTGAAATGTCTGTGACCTTCTGC	62-55 (TD) 1.0 mM	520
Exon 7	GTACCCTTCTGTGGCCCTTCC	AAGGGGCCAAGGCTAATGACC	62-55 (TD) 1.0 mM	343
Exon 8	CAGTGAACTGAGATCGTACCACTGC	AGACAGAAGGCACCCTCCTATGG	66-58 (TD) 3.0 mM, DMSO	601
Exon 9	GTAATGTTGCAGATCCGCAGGC	AGTGGGCCCCTCATTAGACTGC	62-55 (TD) 1.0 mM	466

Hardy-Weinberg equilibrium for all SNPs and sequence variants were tested to eliminate any genotyping error by Chi-square test. The SPSS statistical software was used (SPSS ver11.3; SPSS Inc., Chicago, IL, USA).

2.3.2. Association Analysis: Linakge disequilibrium

For genome-wide association study, the initial analysis was carried out by constructing 2 x 2 tables of the allele counts and 2 x 3 tables of the genotype counts for each SNP in all cases and controls. Subsequently, Pearson's Chi-square statistics were calculated and p-values computed by comparing the χ^2 statistics to a χ^2 distribution with 1 or 2 df for the allelic and genotypic tests, respectively. SNPs yielding a p-value smaller than 2×10^{-7} (Bonferroni adjusted significance of 0.05 [0.05/97,824x2]) were selected for further analysis in the genome-wide association study. In order to avoid spurious associations in genome-wide association study rigorous statistical thresholds are applied. Although bonferroni correction of multiple testing is a conservative approach, smaller p-values generally provide greater support for a true association. Odds ratios, its respective confidence intervals and population attributable risks were calculated accordingly.

Similar statistical approach was applied in the candidate gene screening study. In the candidate gene screening study, the less frequent allele in the control group was considered to be the risk allele. Chi-square test or Fisher's exact test was used in the association analysis to compare the frequencies of various alleles or genotypes between the cases and controls. The OR was calculated by comparing those homozygous for the risk allele (RR) to the baseline group (those homozygous for the normal allele, NN)

(OR_{hom}) and comparing those heterozygous for the risk allele (RN) to the baseline group (OR_{het}). The contrasts for dominant (RR and RN versus NN) and recessive (RR versus RN and NN) effects were also evaluated. Bonferroni method was used for the correction of multiple testing. The SPSS statistical software was used for the calculation (SPSS ver11.5; SPSS Inc., Chicago, IL, USA).

2.3.3. Haplotype Analysis

The SNPHAP (David Clayton's Web site) and the PHASE (Stephens et al., 2001) algorithms were used to construct haplotypes around the region of interesting SNP(s). The Haploxt program (Gonçalo Abecasis' Web site) was used to calculate the *D'*, a standard measure of LD. LD combined case/control samples were then visualized using GOLD (Abecasis and Cookson, 2000). Estimated haplotype frequencies for the case and control groups combined and for each separate group would be calculated. The most probable haplotype pair for each individual was obtained in PHASE (Stephens et al., 2001) and then used to create contingency table and to estimate the effect size of the risk haplotype.

For candidate gene screening, pairwise SNP LD coefficient D' were calculated by Haploview (Barrett et al., 2005). In addition, subsequent haplotype counts between cases and controls were compared and analyzed accordingly.

2.3.4. Interaction Analysis

Gene-environment and gene-gene interaction analysis for AMD were conducted using smoking and *CFH* gene, respectively. Only subjects with smoking status were analyzed which left 143 exudative AMD and 153 controls in the gene-environment interaction analysis. Smoking was coded as "0" for never-smokers and "1" for ever-smokers. The interaction effect of smoking and *HTRA1* was analyzed in a similar fashion as mentioned below.

Six *CFH* SNPs were assessed for their susceptibility with exudative AMD in a previous published study (Chen et al., 2006). In this study, no association of Y402H at *CFH* was found to be associated with exudative AMD. Yet another SNP, rs800292, was identified to be significantly associated with AMD. Hence, this SNP at *CFH* together with the significant SNP at *HTRA1* were evaluated for their interaction effect on exudative AMD. Their joint contribution to AMD predisposition was assessed by logistic regression analysis implemented in R (R statistical analysis package: http://www.r-project.org/), modeling case-control status on *CFH* and *HTRA1* genotypes. A series of models were fitted in order to draw inferences about the most likely and most parsimonious model(s). Both additive (coded 0, 1 and 2 for the number of copies of haplotype) and dominance (coded 1 for possessing exactly one copy of a haplotype and 0 otherwise) effects of each haplotype were considered in the modeling. Models allowing for additive effects (ADD1, ADD2 and ADD1+ADD2), and models incorporate dominance effects (DOM1, DOM2 and DOM1+DOM2) were also fitted. The ADD1 model includes only the additive effects of *HTRA1* at rs11200638 whereas the ADD2 model includes only the additive effects of *CFH* at rs800292. The ADD1+ADD2 models the joint additive effects of

HTRA1 and *CFH*. The DOM1 incorporates dominance effects to ADD1 at rs11200638 of *HTRA1* and the DOM2 model similarly incorporates dominance effects to ADD2 at rs800292 of *CFH*. DOM1+DOM2 models the joint dominance effects of *HTRA1* and *CFH*. Three further interaction models are fitted between *HTRA1* and *CFH*. ADD1*ADD2-INT includes the interactive effects of additive model of *HTRA1* and *CFH*. ADD*DOM-INT includes the interactive effects of additive model of *HTRA1* and *CFH*, additive model of *HTRA1* and dominance model of *CFH*, and dominance model of *HTRA1* and additive model of *CFH*. ALL-INT includes all the interactive effects mentioned and the interactive effect of dominance model of *HTRA1* and dominance model of *CFH*. Models were compared using the Akaike information criterion (AIC) (North et al., 2005). The best fit model will give the smallest AIC. Models were considered to be statistically indistinguishable when AIC differed being <2 units; and the model having fewer parameters was chosen as the best fit and most parsimonious model. The joint ORs of the risk factors were further estimated in this most best fitted and parsimonious model. The baseline haplotype is the observed individuals with the lowest risk genotypes at the two loci studied.

Chapter 3

Results

3.1. Genome-wide Association Study of Exudative AMD

3.1.1. Genotyping and Association Analysis

rs10490924 was the only polymorphism that showed a significant association with AMD using Bonferroni criteria (Table 3.1) out of the 97,824 autosomal SNPs that were informative and passed the quality control checks. The allele frequency χ^2 test yielded a p-value of 4.1×10^{-12} (Figure 3.1A and Table 3.1). The OR was 11.1 (95% confidence interval [CI] 4.83-25.69) for those carrying two copies of the risk allele when compared to wild-type homozygous, but with indistinguishable from unity 1.7 (95% CI 0.75-3.68), for those having a single risk. The risk homozygote accounted for 86% of the population attributable risk (PAR), although this number may be artificial inflated since the risk allele was carried by more than half (~55%) of the AMD cohort (Table 3.1). When likelihood ratio tests were adjusted for gender and smoking status or when genomic control for population stratification, there was little change in significance levels.

SNPS rs10490924 is situated in a hypothetical gene, LOC387715, and resides between two genes on chromosomes 10q26 (Figure 3.1B): *PLEKHA1* encoding a pleckstrin homology domain-containing protein (GenBank ID59338) and *HTRA1* encoding a heat

Figure 3.1 Genome-wide distribution of p-values and characteristics of the associated genome region.

(A) Distribution of p-values for the SNPs in a whole-genome association study of AMD. P-values are plotted as the log10(P) with the SNPs in chromosome order along the x-axis. The dashed horizontal line indicates the Bonferroni adjusted threshold for significant association at the 0.05 level.

(B) A schematic of the genes in the 4-gamete region on chromosome 10q26, as well as the location of SNPs genotyped by microarrays (+) and identified through sequencing (|). SNP rs10490924 is labeled as "8" and rs11200638 is marked with an asterisk. Above is the gene conservation data obtained from the University of California Santa Cruz (UCSC) Golden Path database for the 4-gamete region. The data show the degree of evolutionary conservation among 17 species using the multiz alignment; an increase in the height of the bar indicated an increase in the level of conservation.

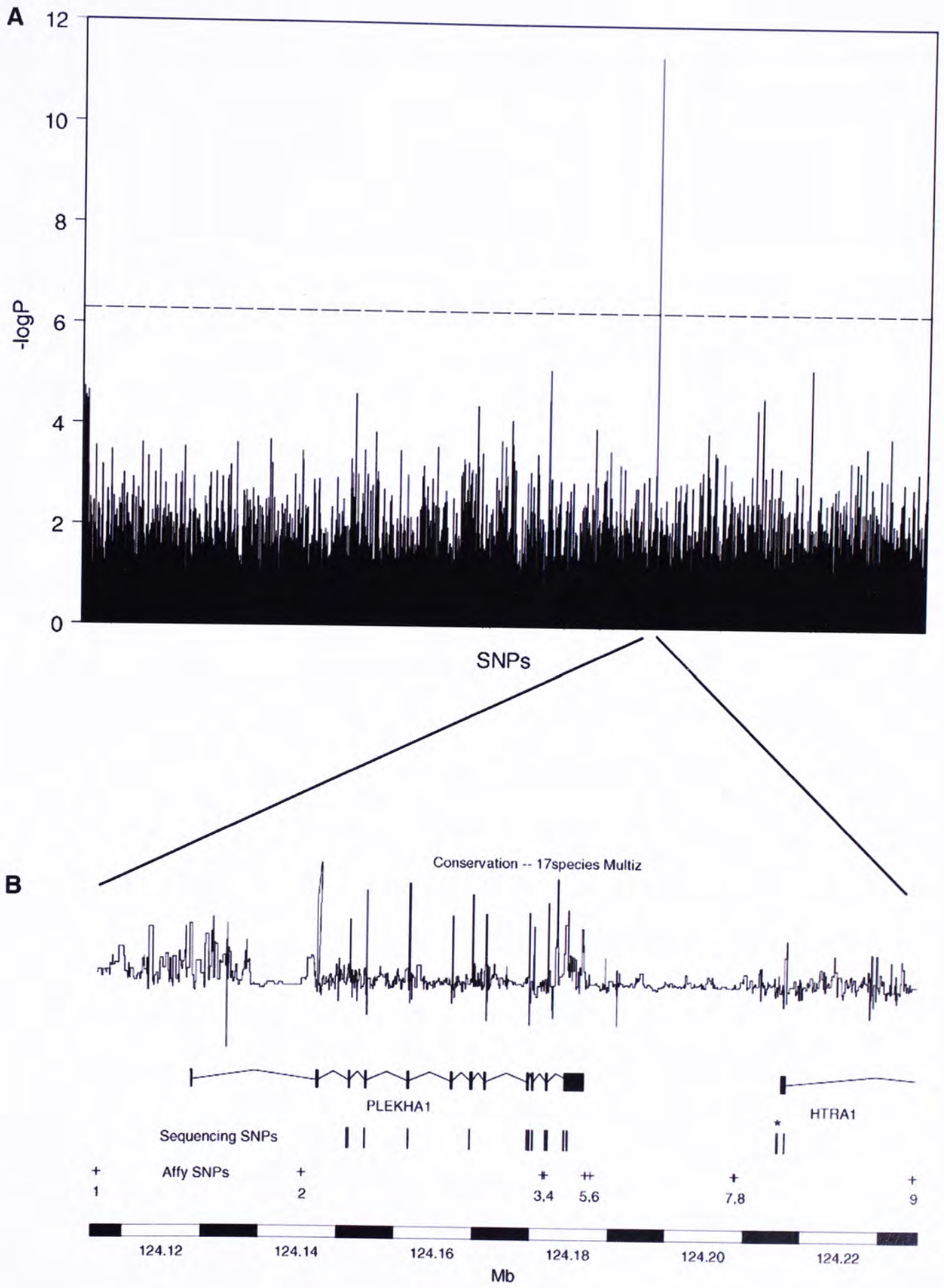

A
12
10
8
6
4
2
0
-logP
SNPs
B
Conservation -- 17species Multiz
PLEKHA1
HTRA1
Sequencing SNPs
Affy SNPs
1
2
3,4
5,6
7,8
9
124.12
124.14
124.16
124.18
124.20
124.22
Mb

Table 3.1 Association, odds ratios and population attributable risk (PAR) for exudative AMD. Odds ratio and PAR compare the likelihood of AMD in individuals with the listed genotype of risk allele versus those homozygous for the wild-type allele.

			Heterozygous risk vs. wild-type homozygotes		Homozygous risk vs. wild-type homozygotes	
SNP (alleles)	**Risk allele**	**Allelic χ^2 nominal P**	**Odds ratio (95% CI)**	**PAR (%) (95% CI)**	**Odds ratio (95% CI)**	**PAR (%) (95% CI)**
rs10490924(G/T)	T	4.08×10^{-12}	1.66 (0.75 – 3.68)	29 (0 – 63)	11.14 (4.83 – 25.69)	86 (69 – 94)
rs11200638(G/A)	A	8.24×10^{12}	1.60 (0.71 – 3.61)	27 (0 – 61)	10.00 (4.38 – 22.82)	84 (66 – 93)

shock serine protease also known as *PRSS11* (GenBank ID 5654). The low sequence homology across species in the intergenic region containing rs10490924 indicates that it is not evolutionarily conserved (Figure 3.1B).

3.1.2. Haplotype Analysis

The region bounded by pairwise SNPs, in which four gametes were observed, was examined to identify the region of interest around rs10490924. This region was being referred to the "4-gamete region". The pattern of linkage disequilibrium (LD) was examined by constructing haplotypes for the seven internal SNPs in the region using SNPHAP (David Clayton's Web site) and the PHASE (Stephens et al., 2001) algorithms. Both algorithms yielded the same haplotypes with similar frequencies. With the construct of haplotypes, the Haploxt program (Gonçalo Abecasis' Web site) was used to calculate the D', a standard measure of LD. LD combined case/control sample were then visualized using GOLD (Abecasis and Cookson, 2000) and was illustrated in Figure 3.2. Estimated haplotype frequencies for the case and control groups combined and for each separate group were given in Table 3.2. The most probable haplotype pair for each individual was obtained from PHASE (Stephens et al., 2001) and then used to determine haplotype counts given in Table 3.3. These haplotype counts were used to create the contingency table and to estimate the effect size of the risk haplotype given in Table 3.4.

To investigate the pattern of LD in this region, the publicly available HapMap database was utilized, which contains information on 45 unrelated Han Chinese individuals from Beijing (CHB). Genotypes for 183 SNPs bound by the 4-gamete region were extracted

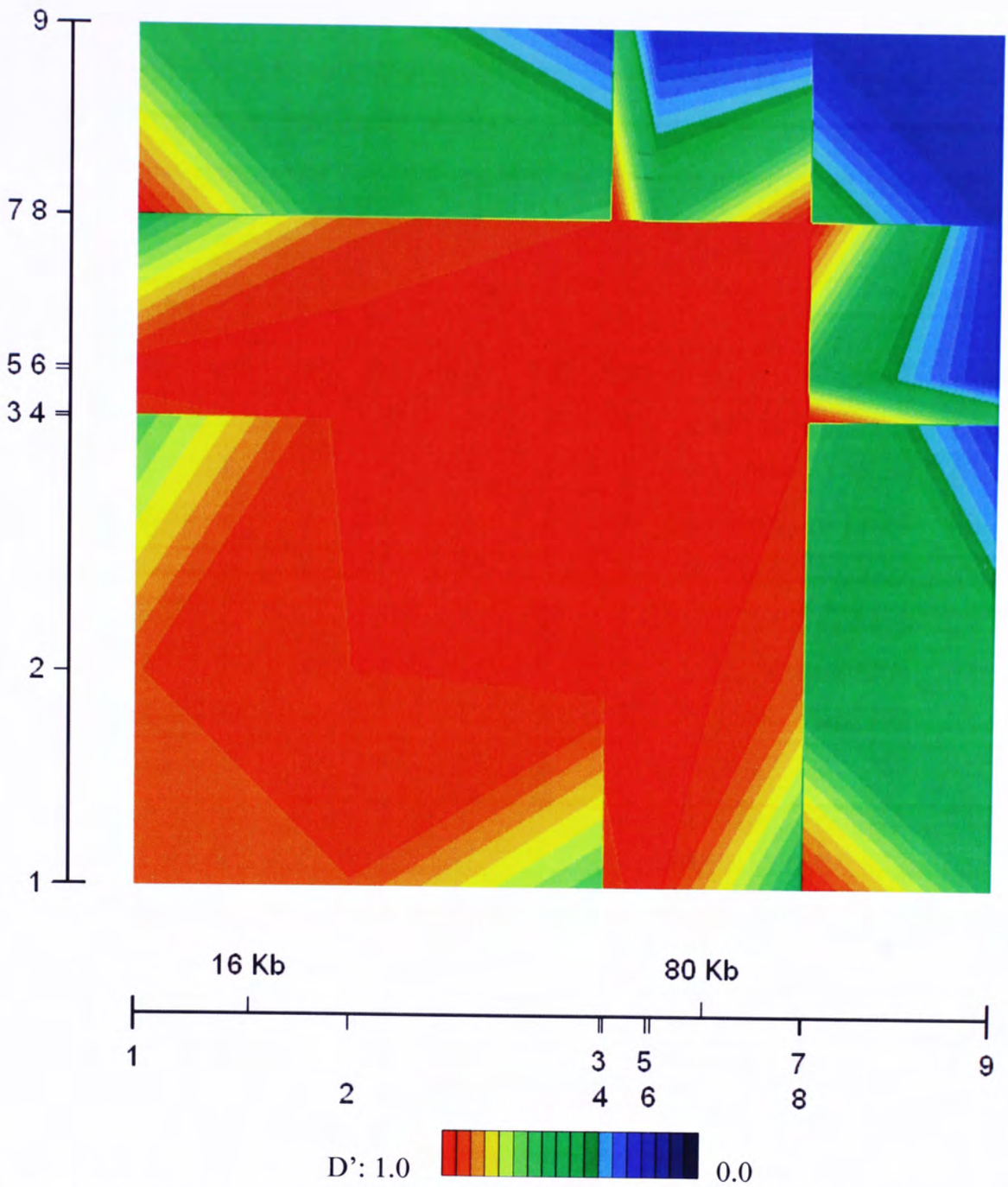


Figure 3.2 Linkage disequilibrium (LD) plot in GOLD of the D' values for the nine SNPs that define the 4 gamete region surrounding rs10490924. Red indicates a *D*' value of 1.0 and Blue indicates a value of 0.0. The marker map is as follows 1 = rs10510109, 2 = rs2421019, 3 = rs2292623, 4 = rs2292625, 5= rs10510110, 6 = rs2280141, 7 = rs2736911, 8 = rs10490924, 9 = rs2284666. Notice that the edge of the region of high LD ends at rs10490924. The seven markers used for the haplotype construction were 2 – 8.

Table 3.2 Haplotype analysis of seven SNPs: rs2421019, rs2292623, rs2292625, rs10510110, rs2282141, rs2736911, rs10790924 in the 4 gamete region. Haplotype frequency estimates as determined by PHASE for the entire population "all" and for the case and control populations separately.

	T/C	A/G	A/G	T/C	T/G	T/C	T/G	ALL	Case	Control
N1	2	1	2	2	2	2	1	0.45743	0.61100	0.34024
N2	2	1	2	2	2	2	2	0.17255	0.09849	0.22908
N3	2	2	2	1	1	1	2	0.14944	0.12047	0.17154
N4	1	1	2	1	1	2	2	0.07105	0.03783	0.09640
N5	1	1	2	1	1	2	1	0.05739	0.05845	0.05659
N6	1	1	1	1	1	2	2	0.03777	0.03009	0.04363
N7	2	2	2	1	1	2	1	0.02841	0.02597	0.03026
N8	2	2	2	1	1	2	2	0.01989	0.01302	0.02514
N9	2	1	2	2	2	1	2	0.00364	0.00021	0.00626
N10	2	2	2	2	2	2	1	0.00188	0.00431	0.00003
N11	1	1	2	1	1	1	2	0.00041	0.00004	0.00068
N12	1	1	1	1	1	2	1	0.00006	0.00006	0.00006
N13	2	1	1	2	2	2	2	0.00002	0.00001	0.00003

Table 3.3 Haplotype counts (and frequency) for cases and controls determined by PHASE using the most probable haplotype pair as the haplotype assignment for an individual.

Haplotype	Case	Control
N1	132 (0.634)	87 (0.355)
N2	17 (0.082)	62 (0.239)
N3	24 (0.115)	45 (0.173)
N4	9 (0.043)	29 (0.112)
N5	6 (0.029)	11 (0.042)
N6	13 (0.063)	10 (0.038)
N7	5 (0.024)	10 (0.038)
N8	1 (0.005)	4 (0.015)
N11	0	1 (0.004)
N9	0	1 (0.004)
N10	1 (0.005)	0

Table 3.4 Contingency table and effect size of the risk haplotype. For the OR and PAR, only the autosomal recessive case is considered, where cases and controls with two copies of the N1 haplotype are compared to those with zero copies.

	Copies of Risk Haplotype (N1)			OR (95% CI)	PAR (95% CI)
	2	1	0		
Case	46 (0.44)	40 (0.39)	18 (0.17)	10.40 (4.68 – 23.14)	0.71 (0.62 – 0.91)
Control	14 (0.11)	59 (0.45)	57 (0.44)		

from the HapMap database. Genotypes were uploaded into Haploview (Barrett et al., 2005) and the LD statistics were calculated. Not all 183 of the HapMap SNPs within in this region were genotyped or passed the default quality control checks (Hardy-Weinberg p-value > 0.01, minimum percentage of genotyped samples >75%, maximum of one mendelian inconsistency and a minimum allele frequency of 0.001). Haplotype blocks were identified according to Gabriel et al. (Gabriel et al., 2002) and illustrated in Figure 3.3.

Among the putative recombination sites revealed by the four-gamete test to surround the marker SNP rs10490924, five major haplotypes, N1 – N5, inferred from nine SNPs (extending 63.9kb, see Figure 3.1), were identified accounting for >90% of all haplotypes in the sample. The odds ratio (OR) for two copies of the risk haplotype, N1, is 10.40, and its 95% confidence interval (CI) overlaps with that of the single SNP rs10490924, 4.68-23.14 vs. 4.83-25.69 (Table 3.1 and Table 3.4). LD was measured and plotted for each pair of the nine SNPs (Figure 3.2). SNP rs10490924 appears to be in LD with the upstream SNPs in *PLEKHA1*, but the next SNP genotyped is too far downstream (26.3kb) to provide meaningful information about recombination/homoplasy breakpoints. The much denser sets of SNPs from the publicly available HapMap database for the Han Chinese in Beijing (CHB) population provided by international HapMap data did not resolve this matter, showing that rs10490924 was not in LD with either gene in the region in this population (Figure 3.3).

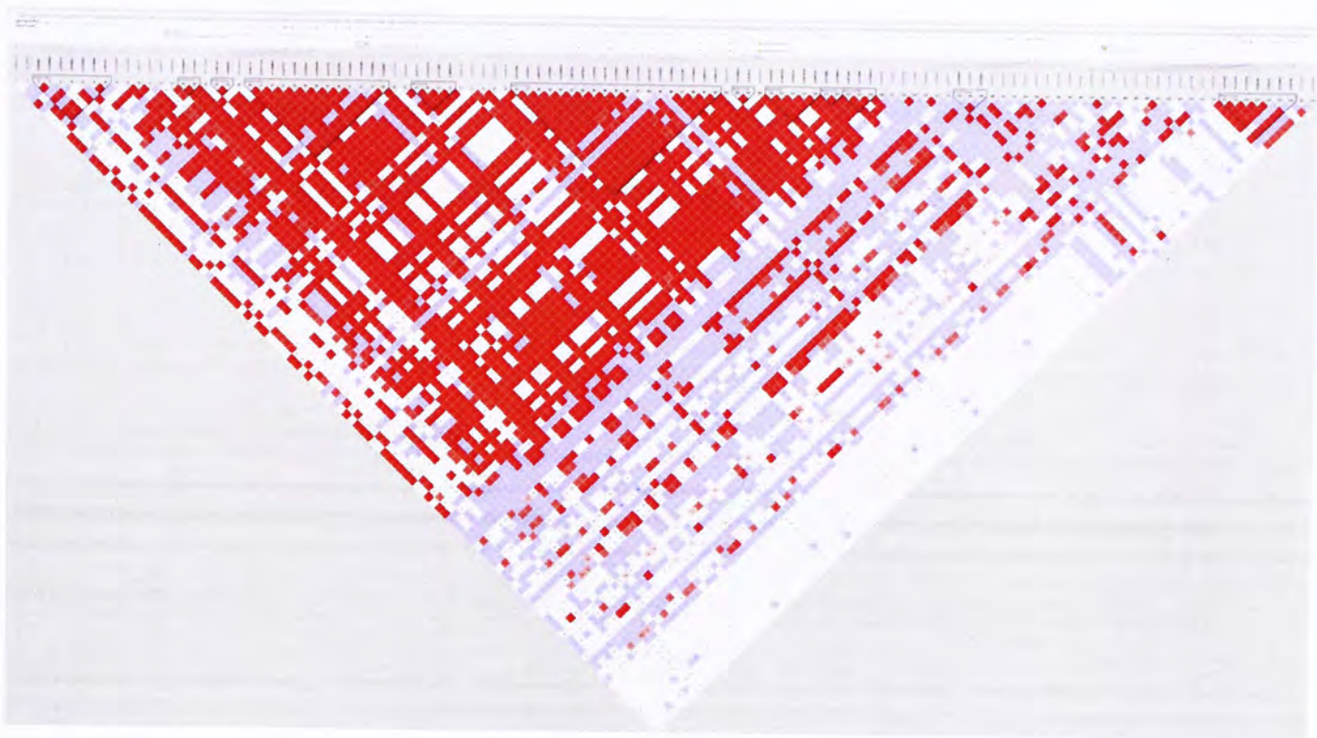


Figure 3.3 Haplotype block definitions across the chromosome 10 region for the publicly available HapMap data from the Chinese population. Using the publicly available HapMap data for the Chinese population, haplotypes and haplotype blocks were computed and visualized in Hpaloview. Dark red indicates higher *D'* values. Light blue indicates high *D'* values, but LOD scores <2. Haplotype blocks as defined by Gabriel et al. are indicated by the black lines. It should be noted that of the 183 HapMap SNPs in this region, only 119 were available for analysis in this population.

HTRA1 gene, lying downstream to LOC387715 (Figure 3.4), was further examined for its association with exudative AMD. The entire genomic region including promoters, exons and intron-exon junctions of *HTRA1*, in search of the functional variant, were subsequently sequenced and presented in the following section.

3.2. HTRA1 Genotpying

The whole gene of *HTRA1* was sequenced by direct sequencing aiming to investigate the existence of genetic variants in *HTRA1* contributing to the risk of exudative AMD. The results are reported in the following section. A total of 163 exudative AMD and 183 controls were screened in the study. The characteristics of the study population were summarized in Table 3.5. The mean ± SD age at AMD diagnosis was 75.5 ± 7.5 years and that of controls was 73.3 ± 6.5. Confounding bias of age was excluded with no age difference being found between the two groups. 54% of the exudative AMD cases were males whereas that of controls was 49.7%. No significant difference was shown in the sex distribution between the two groups (p=0.43). Only 153 exudative AMD cases had smoking status, among which 51.6% were smokers. For the 143 controls with smoking status, only 37.8% of them were smokers. Smoking significantly increased the risk of AMD 1.76-fold (p = 0.017).

3.2.1. Association Analysis

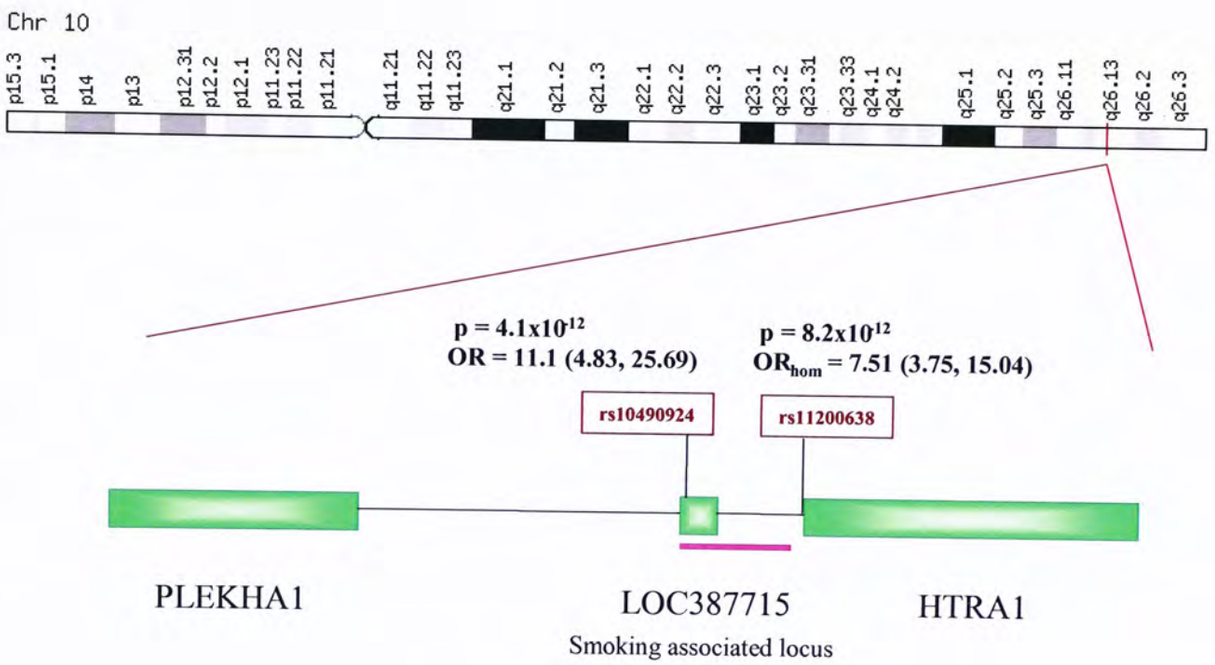


Figure 3.4 Schematic diagram showing location of *PLEKHA1*, *LOC387715* and *HTRA1*. The p-value and OR(95%CI) of the two SNP were obtained from genome-wide association study.

Table 3.5 Characteristics of the study population of *HTRA1* screening

	Exudative AMD	Controls	p-value	OR (95% CI)
Total	163	183		
Males (%)	88 (54.0%)	91 (49.7%)	0.43	
Females (%)	75 (46.0%)	92 (50.3%)		
Mean Age(±S.D.) (Years)	75.5 ± 7.5	73.3 ± 6.5		
Age Range (Years)	60 – 94	60 - 99	0.84	
Smoker	79 (51.6%)	54 (37.8%)	0.017	1.76 (1.11-2.80)
Non-Smoker	74 (48.4%)	89 (62.2%)		
Missing smoking status	10	40		

In the association analysis, the less frequent allele in the control group was considered the risk allele (R) and the other allele was assigned as normal allele (N). A total of 45 sequence variants were identified in *HTRA1* promoter, exons and exon-intron boundaries from 164 exudative AMD and 183 unrelated controls. The genotype frequency and summary of each variant are shown in Table 3.6. Among which 4 SNPs have violated HWE and were excluded for further association and haplotype analysis, namely IVS5+76delGTTT, IVS6+111G>A, IVS7+149C>G and IVS8-36C>T.

For the remaining 41 SNPs, χ^2 statistics of both allele and genotype frequencies were assessed for association to AMD risk. Fifteen variants were found only in one control while six variants existed in only one AMD case. These 21 rare variants were excluded in subsequent association analysis. 340G>C (Arg59Pro) was found heterozygously in both AMD and control each with only one case. This leaves 18 SNPs for further association analysis. Their allele and genotypic frequencies assessment were illustrated with calculated ORs and p-value in Table 3.7 and Figure 3.5. Allele and genotype frequencies of 7 out of the 18 variants were significantly associated conferring exudative AMD (Table 3.7). 162delCinsTCCT was just marginally associated (p=0.001) after Bonferroni correction. This variant was found heterozygously in 2 AMD and 16 controls.

After Bonferroni correction (p =0.05/41 =0.001), four variants still remained significantly associated with exudative AMD. They lie in the promoter and the first exon of *HTRA1*, -491G>A (rs11200638), -359T>C (rs2672598), 230C>T (rs1049331) and 236G>T (rs2293870) with p-value = 5.9×10^{-14}, 5.0×10^{-12}, 1.1×10^{-13} and 1.1×10^{-13},

Table 3.6 *HTRA1* sequence variants identified in exudative AMD and controls

	Location	SNPs	Sequence Variants	Codon Change	Risk Allele	AMD (n = 164)			Control (n = 183)			HWE
						NN	RN	RR	NN	RN	RR	
1	Promoter	rs11200638	-497G>A		A	18 11.0%	51 31.1%	95 57.9%	55 30.0%	90 49.2%	38 20.8%	Conforming
2			-374C>T		C	0 0%	10 6.1%	154 93.9%	0 0%	20 10.9%	163 89.1%	Conforming
3			-369C>T		C	0 0%	4 2.4%	160 973.6%	0 0%	8 4.4%	175 95.6%	Conforming
4		rs2672598	-359T>C		C	1 0.6%	24 14.6%	139 84.8%	18 9.8%	68 37.2%	97 53.0%	Conforming
5	Exon 1		163delCinsTCCT	Leu12leuPro	C	162 98.8%	2 1.2%	0	167 91.3%	16 8.7%	0	Conforming
6			187C>T	Ala24Val	C	125 76.2%	36 22.0%	3 1.8%	25 68.3%	56 30.6%	2 1.1%	Conforming
7			205G>C	Arg26Pro	C	163 99.4%	1 0.6%	0	183 100%	0	0	Conforming
8		rs1049331	230C>T	Ala34Ala	T	18 11.0%	52 31.7%	94 57.3%	55 30.1%	90 49.2%	38 20.8%	Conforming
9		rs2293870	236G>T	Gly36Gly	T	18 11.0%	52 31.7%	94 57.3%	55 30.1%	90 49.2%	38 20.8%	Conforming
10			304G>C	Arg59Pro	-	163 99.4%	1 0.6%	0	182 99.5%	1 0.5%	0	Conforming
11	Intron 1	rs12267142	IVS1-176C>G		C	0	16 9.8%	148 90.2%	0	25 13.7%	158 86.3%	Conforming
12	Intron 2		IVS2+34G>A		-	163 99.4%	1 0.6%	0	183 100%	0	0	Conforming
13			IVS2+81C>T		-	164 100%	0	0	182 99.5%	1 0.5%	0	Conforming
14			IVS2+99T>C		-	163 99.4%	1 0.6%	0	183 100%	0	0	Conforming
15			IVS2+100C>T		C	161 98.2%	3 1.8%	0	175 95.6%	8 4.4%	0	Conforming
16			IVS2+172delACATTCTT		-	163 99.4%	1 0.6%	0	183	0	0	Conforming

Table 3.6 (continued) *HTRA1* sequence variants identified in exudative AMD and controls

	Location	SNPs	Sequence Variants	Codon Change	Risk Allele	AMD (n = 164)			Control (n = 183)			HWE
						NN	RN	RR	NN	RN	RR	
17			IVS2+216A>G		-	164	0	0	182 99.5%	1 0.5%	0	Conforming
18			IVS2+317C>T		T	162 98.8%	2 1.2%	0	182 99.5%	1 0.5%	0	Conforming
19	Exon 3		891G>A	Val221Val	-	164	0	0	182 99.5%	1 0.5%	0	Conforming
20	Intron 3	rs2239586	IVS3+93C>T		C	16 9.8%	77 47.0%	71 43.3%	22 12.0%	92 50.3%	69 37.7%	Conforming
21		rs2239587	IVS3+167G>A		G	16 9.8%	77 47.0%	71 43.3%	22 12.0%	92 50.3%	69 37.7%	Conforming
22	Exon 4		1062C>T	Phe278Phe	-	164	0	0	182 99.5%	1 0.5%	0	Conforming
23	Intron 4	rs2672582	IVS4+99C>T		T	46 28.0%	90 54.9%	28 17.1%	67 36.6%	86 47.0%	30 16.4%	Conforming
24			IVS4-34G>A		-	164	0	0	182 99.5%	1 0.5%	0	Conforming
25	Exon 5		1224A>G		-	164	0	0	182 99.5%	1 0.5%	0	Conforming
26	Intron 5		IVS5+21delG		-	164	0	0	182 99.5%	1 0.5%	0	Conforming
27			IVS5+51G>C		-	164	0	0	182 99.5%	1 0.5%	0	Conforming
28			IVS5+76delGTTT		DEL	105 64%	59 36.0%	0	135 73.8%	48 26.2%	0	Violate
29			IVS5+168C>T		-	164	0	0	182 99.5%	1 0.5%	0	Conforming
30		rs2672583	IVS5+169G>A		A	31 18.9%	90 5.9%	43 26.2%	28 15.3%	87 47.5%	68 37.2%	Conforming
31			IVS5-133G>A		-	163 99.4%	1 0.6%	0	183 100%	0	0	Conforming

Table 3.6 (continued) *HTRA1* sequence variants identified in exudative AMD and controls

	Location	SNPs	Sequence Variants	Codon Change	Risk Allele	AMD (n = 164)			Control (n = 183)			HWE
						NN	RN	RR	NN	RN	RR	
32	Intron 6		IVS6+90G>T		-	164	0	0	182 99.5%	1 0.5%	0	Conforming
33			IVS6+111G>A		G	128 78.0%	32 19.5%	4 2.4%	125 68.3%	57 31.1%	1 0.5%	Violate
34		rs2672585	IVS6+115C>G		G	46 28.0%	92 56.1%	26 15.9%	67 36.6%	88 48.1%	28 15.3%	Conforming
35	Intron 7		IVS7+17C>A		-	162 99.4%	1 0.6%	0	183 100%	0	0	Conforming
36			IVS7+130G>T		-	164	0	0	182 99.5%	1 0.5%	0	Conforming
37			IVS7+149C>G		C	127 77.4%	33 20.1%	4 2.4%	125 68.3%	57 31.1%	1 0.5%	Violate
38			IVS7-123G>C		-	162 99.4%	1 0.6%	0	183 100%	0	0	Conforming
39	Exon 8	rs11538140	1449C>T	Asp407Asp	C	161 98.2%	3 1.8%	0	178 97.3%	5 2.7%	0	Conforming
40			1477G>A	Val417Ile	-	164	0	0	182 99.5%	1 0.5%	0	Conforming
41		rs2272599	IVS8+14G>A		G	28 7.1%	92 56.1%	44 26.8%	31 16.9%	85 46.4%	67 36.6%	Conforming
42			IVS8+61A>G		-	164	0	0	182 99.5%	1 0.5%	0	Conforming
43		rs2293871	IVS8-36C>T		C	62 38.4%	77 47.0%	24 14.6%	57 31.1%	102 55.7%	24 13.1%	Violate
44	Exon 9		1716C>T		-	164	0	0	182 99.5%	1 0.5%	0	Conforming
45			1765C>G		-	164	0	0	182 99.5%	1 0.5%	0	Conforming

Note: Genotypes are ordered: NN – NR – RR, where N is the normal allele and R is the risk allele. The risk allele is defined as the least frequent allele in controls.

Table 3.7 Allelic and genotypic significant assessment of *HTRA1* variants in exudative AMD

	HTRA1	Sequence Variants	Allele			Genotype
			OR	95% CI	p-value	p-value
1	rs11200638	-497G>A*	3.337	2.423-4.596	$5.9x10^{-14}$*	$2.97x10^{-12}$*
2	-	-374C>T	1.838	0.848-3.987	0.12	0.11
3	-	-369C>T	1.829	0.54-6.189	0.39	0.32
4	rs2672598	-359T>C*	4.611	2.909-7.307	$5.0x10^{-12}$*	$5.04x10^{-10}$*
5	-	163delCinsTCCT^	7.451	1.7-32.658	0.001^	0.001^
6	-	187C>T	1.335	0.872-2.044	0.18	0.17
7	rs1049331	230C>T*	3.286	2.387-4.522	$1.14x10^{-13}$*	$5.66x10^{-12}$*
8	rs2293870	236G>T*	3.286	2.387-4.522	$1.14x10^{-13}$*	$5.66x10^{-12}$*
9	rs12267142	IVS1-176C>G#	2.023	1.056-3.873	0.031#	0.22#
10	-	IVS2+100C>T	3.631	0.766-17.225	0.11	0.23
11	-	IVS2+317C>T	2.239	0.202-24.81	0.60	0.60
12	rs2239586	IVS3+93C>T	1.188	0.869-1.624	0.28	0.53
13	rs2239587	IVS3+167G>A	1.188	0.869-1.624	0.28	0.53
14	rs2672582	IVS4+99C>T	1.209	0.894-1.635	0.22	0.22
15	rs2672583	IVS5+169G>A#	1.347	0.996-1.822	0.053#	0.09#
16	rs2672585	IVS6+115C>G	1.207	0.892-1.633	0.22	0.22
17	rs11538140	1449C>T	1.500	0.356-6.328	0.73	0.58
18	rs2272599	IVS8+14G>A	1.225	0.906-1.656	0.187	0.12

* These 4 SNPs remain significantly associated with AMD after Bonferroni correction (0.05/18/2=0.001).
^ This variant is marginally significant with 2 heterozygous carriers in AMD and 16 hetrozygous carriers in controls.
# These 2 SNPs become insignificantly associated with AMD after Bonferroni correction.

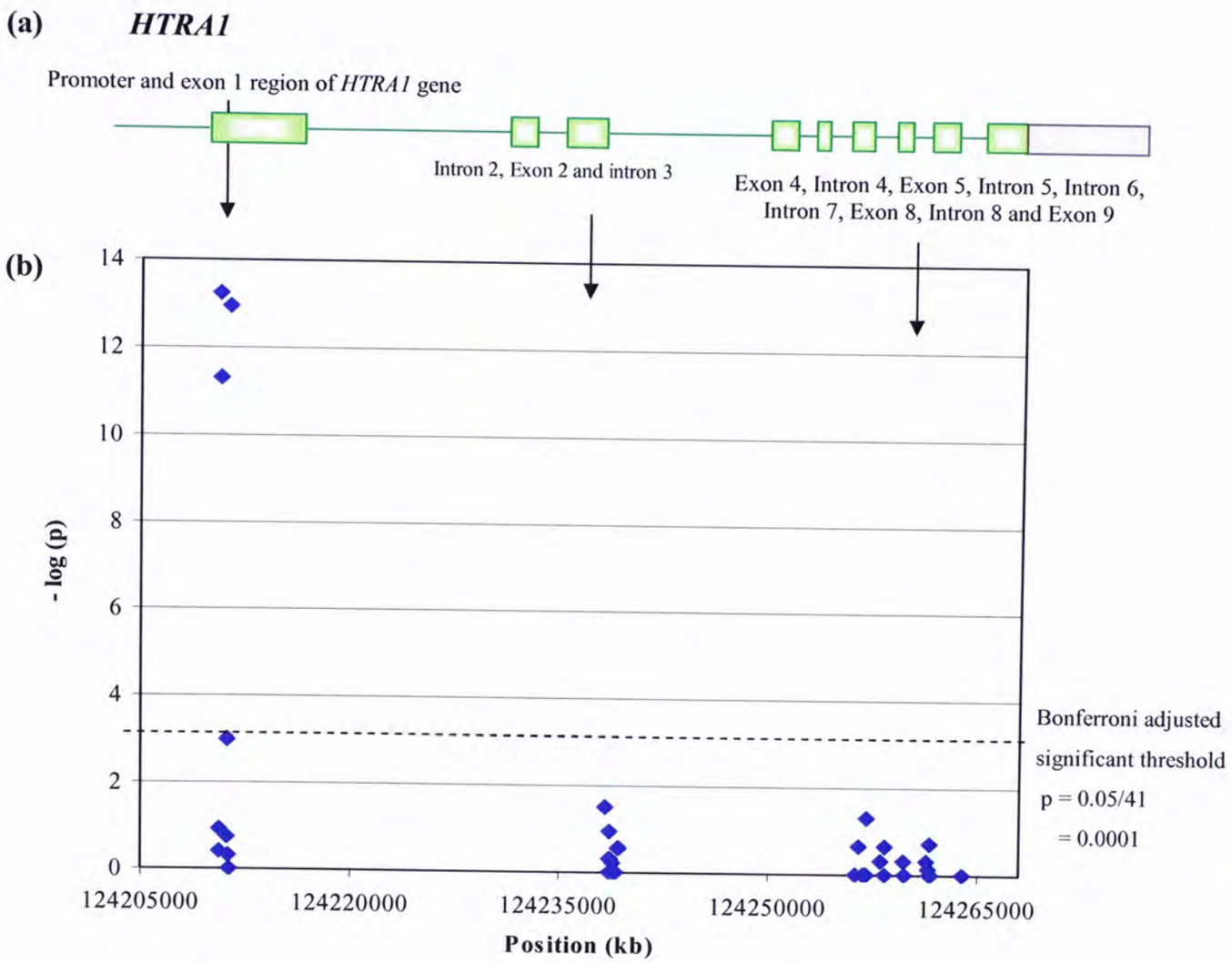


Figure 3.5 Schematic view of the association of exudative AMD with 41 variants across a 54 Mb region on chromosome 10 in *HTRA1* gene. The position for each SNP is given on a kilobasepair scale. (a) Location of exons of *HTRA1* that map to the interval. (b) –log (p) is plotted against the chromosomal location of the 41 variants identified.

respectively. 230G>T and 236G>T are in perfect LD, where D'=1 and are synonymous variants with no amino acid change (Ala34Ala and Gly36Gly, respectively). The magnitude of the effect of these 4 extremely significant variants was estimated by odds ratio (ORs) in different models (dominant, recessive, heterozygote and homozygote) and population attributable risks (PARs). Table 3.8 summarizes the analyzed effects. Both dominant and recessive models showed significant association with AMD in all of these 4 SNPs. In addition, an allele-dosage effect appeared to be present in all these 4 SNPs. Carriers of two risk alleles were at substantially higher risk (about 4 times higher) to exudative AMD than are carriers with one risk allele, the OR_{het} and OR_{hom} for the 4 SNPs were 1.73 vs. 7.64, 6.35 vs. 25.79, 1.76 vs. 7.56 and 1.76 vs. 7.56, respectively (Table 3.8). Two significant SNPs were in the promoter region, -497G>A and –359T>C and the other 2 SNPS were in exon 1 of *HTRA1*. With the observed increased risk in carriers of the two risk alleles of these SNPs, the PARs were also found to have increased in similar fashion. PAR estimates at –497G>A, 230C>T and 236G>T increased from 24% to 55% with heterozygous and homozygous genotypes, respectively (Table 3.8). Relative high frequency of the risk (C) genotype at –359T>C, rs2672598, in the case/control sample (AMD: 84.8%; Controls: 53.0%) was observed suggesting an overestimation of the corresponding attributable risk, and so does not provide a good estimate of the risk in the population. The OR of this SNP was also inflated with OR_{het} being 6.35 95%CI (0.08-50.18) and OR_{hom} being 25.79 95%CI (3.39-196.46). The observed relatively high frequency of the risk genotype was TT: 0.6%; TC: 14.6%; CC: 84.8% for exudative and TT: 9.87%; TC: 37.2%; CC: 53.0% for controls. The single SNP association analysis revealed no other interesting variants but the variant found in

Table 3.8 Estimated ORs, p-value, PARs, and corresponding 95% CI of *HTRA1* gene variants.

Gene Variants	Dominant (RN+RR vs. NN)			Recessive (RR vs. RN+NN)			Heterozygote (RN vs. NN)			Homozygote (RR vs. NN)		
	OR_{dom} (95% CI)	p-value	PAR%	OR_{rec} (95% CI)	p-value	PAR%	OR_{het} (95% CI)	p-value	PAR%	OR_{hom} (95% CI)	p-value	PAR%
-497G>A	3.48 (1.95-6.242)	$1.34x10^{-5}$	47.8	5.25 (3.27-8.43)	$1.17x10^{-12}$	31.8	1.73 (0.92-3.26)	0.088	23.5	7.64 (3.98-14.65)	$1.10x10^{-10}$	55.0
-359T>C	12.54 (1.65-95.30)	0.002	86.4	4.93 (2.94-8.25)	$2.44x10^{-10}$	52.35	6.35 (0.080-50.18)	0.048	76.6	25.79 (3.39-196.46)	$6.18x10^{-6}$	90.4
230C>T	3.48 (1.95-6.242	$1.34x10^{-5}$	47.8	5.12 (3.19-8.22)	$2.52x10^{-12}$	31.1	1.76 (0.94-3.32)	0.076	24.3	7.56 (3.94-14.51)	$1.44x10^{-10}$	54.9
236G>T	3.48 (1.95-6.242	$1.34x10^{-5}$	47.8	5.12 (3.19-8.22)	$2.52x10^{-12}$	31.1	1.76 (0.94-3.32)	0.076	24.3	7.56 (3.94-14.51)	$1.44x10^{-10}$	54.9

Note: Genotypes are ordered: NN – NR – RR, where N is the normal allele and R is the risk allele. The risk allele is defined as the least frequent allele in controls.
NN: Homozygous normal alleles
RR: Homozygous risk alleles
RN: Heterozygous genotype

the promoter, -497G>A, which was the same SNP (rs11200638) identified in the whole genome association study.

3.2.2. Haplotype Analysis

LD analysis revealed three regions of linkage disequilibrium in *HTRA1* gene (Figure 3.6). Three haplotype blocks were constructed with Haploview (Barrett et al., 2005). Only the first haplotype block that lies in the promoter and exon one region was significantly associated predisposing exudative AMD. Five SNPS are within the block namely, -491G>A (rs11200638), -359T>C (rs2672598), 187C>T, 230C>T (rs1049331) and 236G>T (rs2293870). The haplotype frequencies of the block were shown in Table 3.9 with ACCTT and GTCCG being significant AMD-predisposing haplotypes with p= 6.68×10^{-14} and 4.97×10^{-12}, respectively. This haplotype block contains the four significant variants identified in the single SNP association analysis mentioned earlier. After permutation, these two haplotypes remained exceptionally significant, further supported this region being associated to exudative AMD as in single SNP analysis. Haplotype block 2 contains only 2 SNPs that are in intron 3. Haplotype block 3 spans from intron 4 to intron 8. Both haplotype block 2 and 3 were not associated with risk of exudative AMD.

3.2.3. rs11200638 – Smoking Interaction

Logistic regression modeling was used to build a model of the joint contribution of *HTRA1* and cigarette smoking, and *HTRA1* and *CFH*. rs11200638 of *HTRA1* and rs800292 of *CFH* were chosen for the interaction assessment under the criteria that they

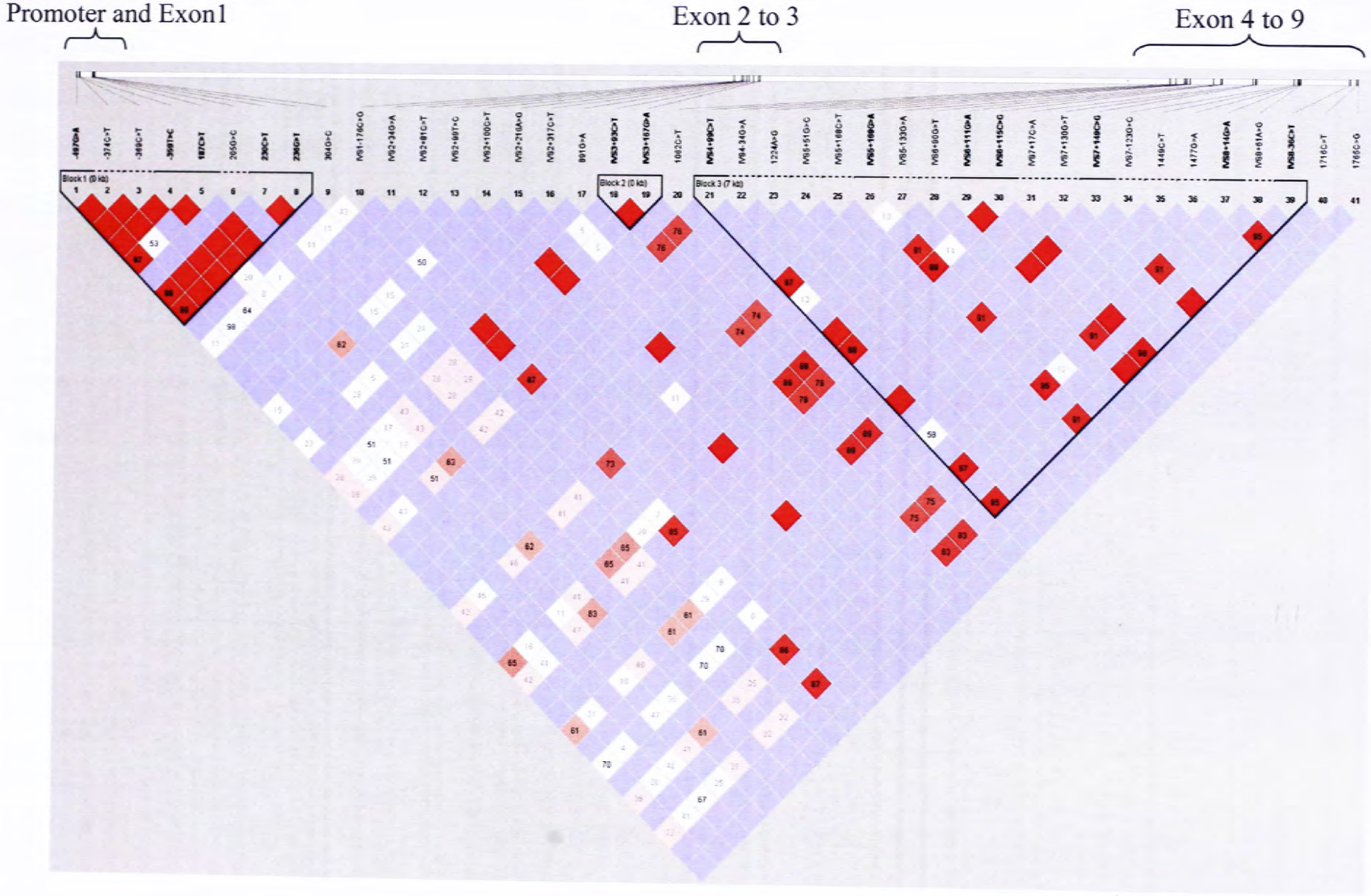


Figure 3.6 Haploview plot depicting the haplotype block structure of the exudative AMD-associated region. The relative choromosomal position of each SNP is given in the upper diagram.

Table 3.9 Haplotype block analysis of *HTRA1* on risk of exudative AMD by Haploview.

Haplotype Block	Haplotype Frequency	AMD:Control Ratios	Chi Square	p-value	p-value$_{corr}$	OR
Block 1: -497G>A, -359T>C, 187C>T, 230C>T, 236G>T						
ACCTT*	0.584	240:88, 165:201	56.16	6.68×10^{-14}*	<0.000001*	3.32 (2.41-4.57)
GTCCG*	0.187	16:302, 104:262	47.699	4.97×10^{-12}*	<0.000001*	0.13 (0.077-0.23)
GCTCG	0.146	41:287, 60:306	2.109	0.15	0.94	
GCCCG	0.079	20:308, 35:331	2.847	0.09	0.74	
Block 2: IVS3+93C>T, IVS3+167G>A						
CG	0.647	219:109, 230:136	1.168	0.28	0.99	
TA	0.353	109:219, 136:230	1.168	0.28	0.99	
Block 3: IVS4+99C>T, IVS5+169G>A, IVS6+111G>A, IVS6+115C>G, IVS7+149C>G, IVS8+14G>A, IVS8-36C>T						
TAGGCGC	0.409	142.9:185.1, 140.9:225.1	1.839	0.1751	0.9598	
CGGCCAT	0.240	76.9:251.1, 89.9:276.1	0.119	0.7296	1.0000	
CGGCCAC	0.184	56.4:271.6, 71.1:294.9	0.582	0.4457	1.0000	
CGACGAT	0.132	36.8:291.2, 55:311	2.198	0.1382	0.9297	

*These two genotypes of haplotype block 1 were significantly associated with risk of AMD.
p-value$_{corr}$ is the corrected p-value after permutation.

were the most significant SNPs associated with exudative AMD in single SNP analysis. The risk G allele of rs800292 was found to be at 2.5 times higher risk to AMD in our previous study (Chen et al., 2006). Table 3.10 summarizes the best fit and parsimonious model analyzed by logistic regression.

Smoking is one of the most significant environmental risk factor for AMD. Results from logistic regression suggested that the joint effects of smoking and rs11200638 of *HTRA1* was best described by independent multiplicative effects, without significant dominance nor interacting effects (Table 3.10). Independent multiplicative effect is the additive effect on the log-scale. This model yielded the lowest AIC (AIC=367.5). The second best fit model was the independent multiplicative effect of rs11200638 at *HTRA1* alone with no smoking effect and yielded an AIC of 369.9. The AIC difference between these two models was 2.4 (AIC diff > 2); hence, by definition the most best fitted and parsimonious model was the joint independent multiplicative effect (or independent log-additive effect) of smoking and rs11200638 of *HTRA1*.

Given the evidence of independent multiplicative joint effects of smoking and *HTRA1*, the combined effect of smoking and *HTRA1*-rs11200638 was further illustrated by the joint ORs shown in Table 3.11a and b. The risk of AMD due to the risk genotype was increased with smoking. Estimates from this model demonstrated a 15.71 fold increased risk to exudative AMD in homozygote carriers of the *HTRA1*-risk allele who were ever-smokers compared with non-smokers with no risk alleles at *HTRA1*-rs11200638. The heterozygote ever-smoking carrier of the *HTRA1*-risk allele was 4.80 times more likely to be exudative AMD. The adjusted OR of smoker with non-risk allele was calculated to

Table 3.10 Results of fitting two factor models by logistic regression.

	Two-Factor Model	AIC	AIC Difference
	HTRA1:rs11200638 (Factor 1) and Smoking (ever vs. never)		
Single	ADD1[#]	369.9	2.4[~]
Single	SMOKE	408.2	40.7
Single	DOM1	405.9	38.4
Joint	ADD1 - SMOKE[#]	367.5	0[#]
Joint	DOM1 - SMOKE	402.7	35.2
Interaction	ADD*SMOKE-INT	400.1	32.6
Interaction	DOM*SMOKE-INT	413.8	46.3
Interaction	ADD-DOM-SMOKE-INT	402.1	34.6
	HTRA1:rs11200638 (Factor 1) and CFH:rs800292 (Factor 2)		
Single	ADD1	432.2	13.2
Single	ADD2	466.7	47.7
Single	DOM1	471.0	52.0
Single	DOM2	476.1	57.1
Joint	ADD1 + ADD2[#]	419.0	0[#]
Joint	DOM1 + DOM2	465.5	46.5
Interaction	ADD1*ADD2-INT	461.5	42.5
Interaction	ADD*DOM-INT	448.9	29.9
Interaction	ALL-INT	448.7	29.7

AIC difference is the difference from the AIC of the best fitting model.

[#] is the most parsimonious and best fit model in both interaction analysis. Model with best fit (lowest AIC) has AIC difference = 0.

[~] is the second best-fit model and gives an AIC difference of 2.4. This model is contributed by the multiplicative effect of *HTRA1* only.

Table 3.11a Genotype distribution of rs11200638 in HTRA1 and smoking in exudative AMD (ever vs. never smoked).

HTRA1 (rs11200638)	Smoking status			
	Never	Ever	Never	Ever
	Controls (n=143)		Exudative AMD (n=153)	
GG	30 (2.1%)	15 (10.5%)	6 (3.9%)	11 (7.2%)
GA	44 (30.8%)	25 (17.5%)	25 (16.3%)	24 (15.7%)
AA	15 (10.5%)	14 (9.8%)	43 (28.1%)	44 (28.8%)

Table 3.11b Joint ORs and 95% CI of rs11200638 in HTRA1 and smoking.

	OR (95% CI)		
HTRA1 rs11200638	$n_{controls} = 143$ $n_{AMD} = 153$	**Smoking status**	
		Never	**Ever**
Main Effects	OR_{smoke}	1.0 (Ref)	1.76 (1.11-2.80)
	PAR_{smoke}	12.2	
	PAR_{HTRA1}	53.1	
	PAR_{Joint}	68.7	
	OR_{HTRA1}	**Joint effects**	
GG	1.00 (Ref)	1.00 (Ref)	3.67 (1.14-11.84)
GA	1.88 (0.96-3.66)	2.84 (1.04-7.76)	4.80 (1.70-13.58)
AA	7.94 (3.95-15.97)	14.33 (4.99-41.18)	15.71 (5.43-45.49)

be 3.67. This indicated about 5-fold increase risk of smoker with homozygous risk allele than that with no risk allele (Table 3.11b). Corresponding ORs for smoker and non-smoker homozygous risk-allele carrier of *HTRA1*-rs11200638 were similar (15.71 and 14.33, respectively). This indicated that the risk allele of *HTRA1*-rs1120638 exerted a stronger effect on AMD-risk than the effect of smoking.

Population attributable risk (PAR) percentage was calculated to be 12.2% for smoking, 53.1% for *HTRA1*, and 68.7% for the joint gene-environment effect (Table 3.11b). There was an observable increase PAR (about 5.5-fold increased) of the joint effect of smoking and *HTRA1* risk-allele, indicating homozygote risk allele smoker has a substantial higher likelihood of developing exudative AMD in the population basis.

3.2.4. rs11200638 – rs800292 Interaction

Gene-gene interaction analysis on different models of the two variants on *HTRA1* and *CFH* showed no evidence of interaction in developing exudative AMD. These two variants contributed independently to the disease risk. The best fit and most parsimonious model for their joint effect was best described by independent multiplicative model (independent log-additive effect) with no dominance nor interaction effects, yielding the lowest AIC of 419.0 (Table 3.10). The second best fit model only involved the main additive effect of *HTRA1* alone (single gene effect) and gave an AIC of 432.2. The AIC difference between these two models was much greater than 2 (AIC difference = 13.2 as shown in Table 3.10). By definition, this model was not better than the lowest AIC model. The interaction analysis suggested the two genes

together contributed to the disease risk in an independent multiplicative manner, corresponding to a linear additive model on a logarithmic scale, with no interaction being the best-fitted and parsimonious model.

Given the evidence of independent multiplicative effect of the two variants in *HTRA1* and *CFH* observed in the model comparison, corresponding ORs of AMD for each possible combination of the genotypes from the two variants were estimated and shown in Table 3.12a and b. The risk allele of rs11200638 at *HTRA1* was the A allele where as that of rs800292 at *CFH* was the G allele. The ORs were compared with the baseline genotype of the two genes that showed the lowest frequency of the disease risk alleles (homozygote G carrier of *HTRA1* with homozygote A carrier of *CFH*). Frequency of homozygous risk genotype at both loci was 5 times lower in the control (6.56%) than in the cases (34.36%) as shown in Table 3.12a. A joint disease odds ratio of 23.3 for individuals with homozygous risk alleles at both loci was observed when compared with the baseline wild-type (non-risk) genotype (Table 3.12b), with a wide range of calculated 95%CI being 2.49 to 218.24. Individuals being homozygote for *HTRA1*-risk (A) allele and any genotype for *CFH*-risk allele (for the AA-GA joint genotype, OR 7.63; AA-AA joint genotype, OR 6.43) were at higher AMD risk than those being homozygote for *CFH*-risk allele and any genotype for *HTRA1*-risk allele (for the GA-GG joint genotype, OR = 3.95; and GG-GG joint genotype, OR = 2.63) (Table 3.12b). The homozygous risk genotype of *HTRA1* appeared to exert 2-fold stronger risk than that of *CFH* in the joint multiplicative model conferring exudative AMD. The *HTRA1*-risk allele revealed a stronger impact to the development of exudative AMD than *CFH*-

Table 3.12a Genotype distribution of rs11200638 in *HTRA1* and rs800292 in *CFH* in exudative AMD.

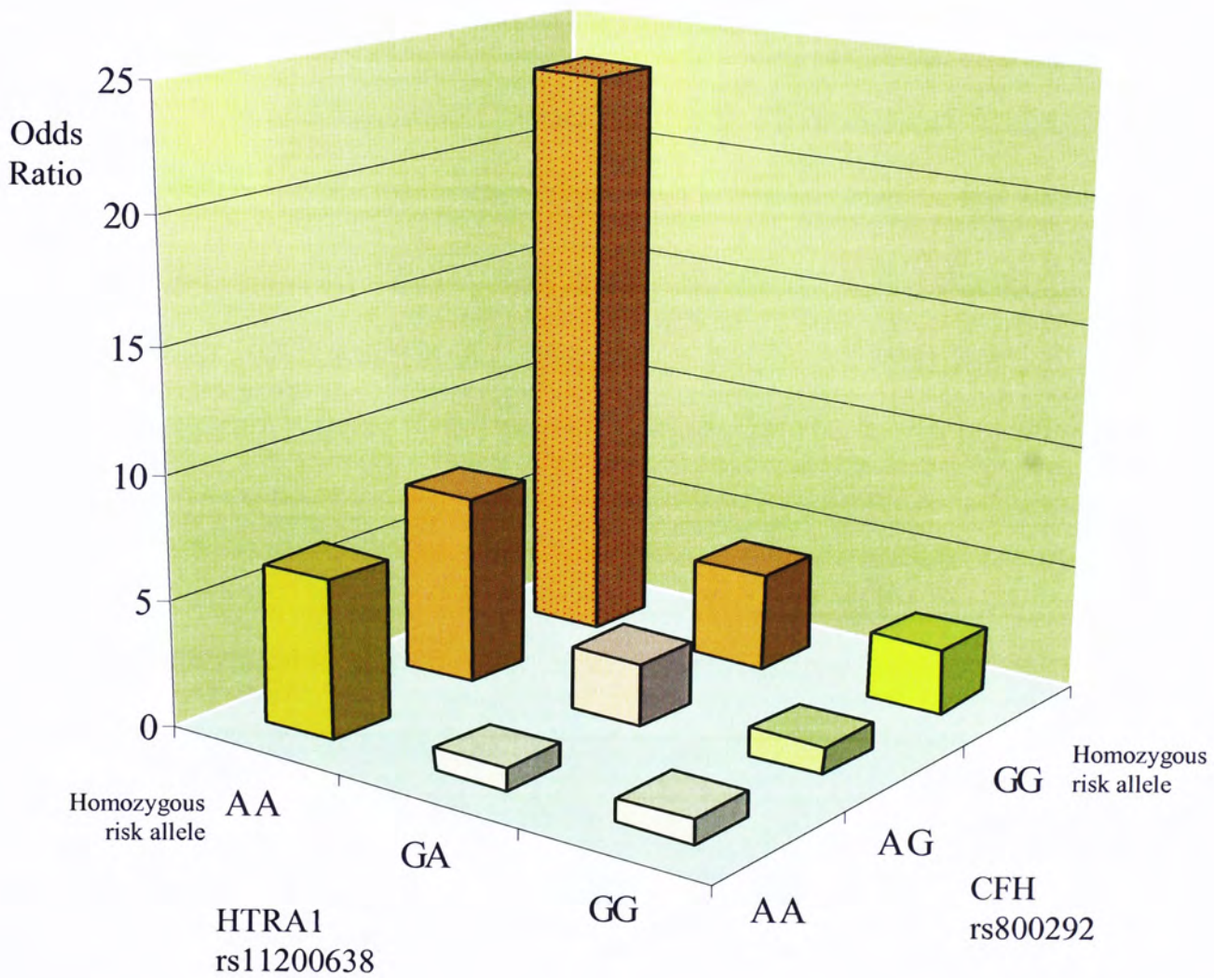
Genotype at HTRA1 (rs11200638)	Genotype at rs800292 in CFH					
	Controls (n = 183)			AMD (n = 163)		
	AA	AG	GG	AA	AG	GG
GG	5 (2.73%)	31 (16.9%)	19 (10.38%)	1 (0.61%)	7 (4.29%)	10 (6.13%)
GA	17 (9.29%)	35 (19.13%)	38 (20.77%)	3 (1.84%)	18 (11.04%)	30 (18.40%)
AA	7 (3.83%)	19 10.38%)	12 (6.56%)	9 (5.52%)	29 (17.79%)	56 (34.36%)

Table 3.12b Joint ORs and 95% CIs of rs800292 in *CFH* and rs11200638 in *HTRA1*

Main Effects	OR & 95% CI for			
	$n_{Controls}$=183	CFH rs800292		
	n_{AMD}=163	AA	AG	GG
	OR_{CFH}	1.0 (Ref)	1.09 (0.60-1.97)	2.14 (1.18-3.86)
	PAR_{CFH}	41.2		
	PAR_{HTRA1}	54.9		
HTRA1 rs11300638	PAR_{Joint}	78.4		
	OR_{HTRA1}	Joint Effects		
GG	1.00 (Ref)	1.00 (Ref)	1.13 (0.11-11.24)	2.63 (0.27-25.71)
GA	1.88 (0.96-3.66)	0.88 (0.074-10.46)	2.57 (0.28-23.70)	3.95 (0.44-35.62)
AA	7.94 (3.95-15.97)	6.43 (0.61-68.31)	7.63 (0.83-70.52)	23.3 (2.5-218.2)

risk allele. The plot of the two-locus genotype specific AMD risks on Figure 3.7 further illustrates the stronger impact of *HTRA1* on our study exudative AMD subjects. Population attributable risk percentage was estimated to be 41.2% for *CFH*-rs800292, 54.9% for *HTRA1*-rs11200638, and 78.4% for the joint effect as shown in Table 3.12b.

Figure 3.7 Two-locus (*HTRA1* and *CFH*) genotype specific AMD risks.



Chapter 4

Discussion

4.1. Genome-wide Association Study of Exudative AMD

Previous studies showed that CFH is a risk factor of AMD predominantly in Caucasians and not in Chinese and Japanese. The racial difference in prevalence of the C allele at Y402H in CFH between Chinese/Japanese and Caucasians lead to the postulation of another common genetic variant(s) in the genome giving rise to this complex disorder as suggested by the CDCV hypothesis. Genome-wide association study is an effective and powerful approach to explore on the existence of other genetic factors contributing to the development of AMD.

In this genome-wide association study, a new genetic variant, rs10490924, located on chromosome 10q26 was successfully mapped to the risk of exudative AMD using only about 100 cases and controls. This variant attributes to the population with large associated PAR of 84%. The impact of this genetic variant was extremely high conferring an OR of 11.1 exudative AMD. The extreme high PAR and OR identified conferring to the disease allow a small study group for this successful association.

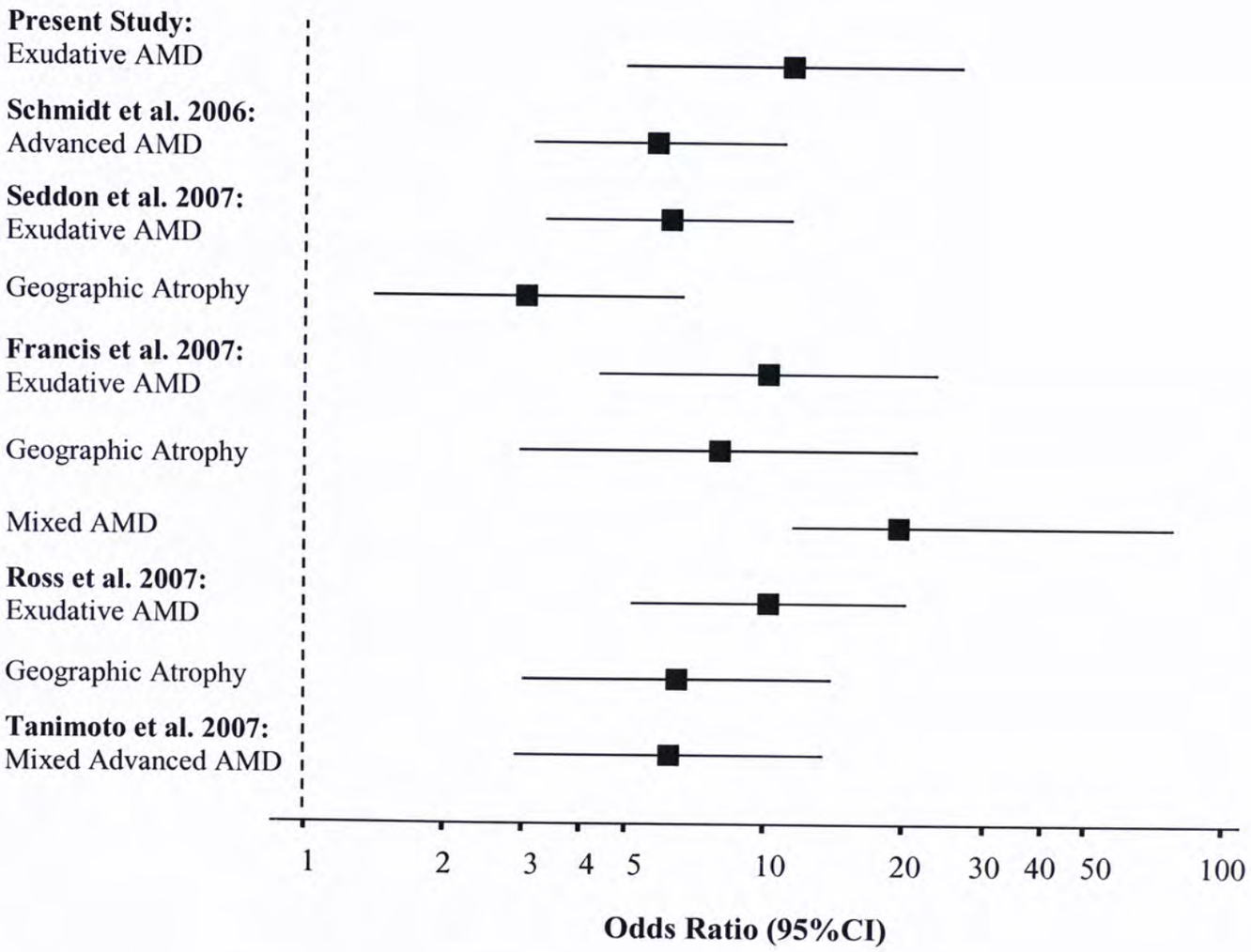
This study had demonstrated the outstanding power of genome-wide association approach to identify disease susceptibility genes for complex genetic disease. In contrast to candidate genes studies, which select genes for study based on known or suspected disease mechanisms, genome-wide association studies permit a comprehensive scan of the genome in an unbiased fashion and thus have the potential to identify novel susceptibility regions (loci). Family-linkage based approaches only consider meiotic recombination events in the families studied while genome-wide association studies investigate that in a population-based. With this approach, association signals are localized to small regions of the chromosome containing only a single to a few genes, enabling rapid detection of the actual disease susceptibility gene.

rs10490924 lies in a hypothetical gene, LOC387715, residing between *PLEKHA1* and *HTRA1* (Figure 3.5). LOC387715 has no evidence of known biological function. It is identified to be highly expressed in placental tissue and an extremely low expression in the retina (Jakobsdottir et al., 2005;Rivera et al., 2005). After publication of this wide-genome association data in 2006, numerous studies consistently replicated the association of this susceptible SNP to risk of AMD in both exudative AMD and geographic atrophy (GA). The relative risk of LOC387715-rs10490924 and AMD found in each subsequent study was ranged from 3.0 with GA to 29.3 with mixed advanced AMD as shown in Table 4.1 and Figure 4.1. Comparing with other subsequent studies, the present study yielded the highest risk (OR=11.14) to exudative AMD. The risk of this SNP to GA yielded a much lower risk ranging from 3.0 to the maximum risk being 7.9-fold (Francis et al., 2007). The mixed advanced AMD has yielded the highest risk of

Table 4.1 Comparisons of the odds ratio of LOC387715 at rs10490924 to the risk advanced AMD among the present study and subsequent follow-up studies by other groups.

AMD Grade	OR_{hom}	95% CI	Cases	Controls	References
Exudative AMD	11.14	4.83-25.69	96	130	Present study
Advanced AMD	5.73	3.07-10.71	610	259	(Schmidt et al., 2006)
Exudative AMD	6.1	3.3-11.2	122	463	(Seddon et al., 2007)
Geographic Atrophy	3.0	1.4-6.5	69	463	
Exudative AMD	10.0	4.3-23.3	241	280	(Francis et al., 2007)
Geographic Atrophy	7.9	2.9-21.0	147	280	
Mixed advanced AMD	29.3	11.4-75.6	142	280	
Exudative AMD	10.12	5.12-20.01	227	329	(Ross et al., 2007)
Geographic Atrophy	6.43	3.00-13.79	172	329	
Mixed advanced AMD	6.20	2.87-13.40	95	99	(Tanimoto et al., 2007)

Figure 4.1 Schematic comparisons of the association of rs10490924 at LOC387715 to the risk of age-related macular degeneration among the present study and follow-up studies by other groups. The odds ratios (OR) of homozygotes for each study corresponds to the center of the box, and the 95% confidence intervals (CI) are represented by the horizontal lines through the boxes.



29.3 with a wide range of 95% CI (11.4-75.6). These subsequent studies further supported the finding of rs10490924 at LOC387715 being associated not only with exudative AMD but also with geographic atrophy.

The frequency of the risk T alleles at rs10490924 was higher in exudative AMD than in controls, as was found in Caucasians (Francis et al., 2007;Ross et al., 2007;Schmidt et al., 2006;Seddon et al., 2007;Shuler, Jr. et al., 2007) and Japanese (Tanimoto et al., 2007). Since this hypothetical gene has no known biological function, further study is required to determine its biological function so as to assess its role in the etiology of exudative AMD. This SNP can also be a surrogate SNP. Variants in LD with this SNP need to be evaluated for their association with exudative AMD. The frequency of the risk allele at *PLEKHA1*, located near to LOC387715, has reported to exhibit no difference in the Caucasians (Fisher et al., 2006;Rivera et al., 2005). Hence, the other nearby gene, *HTRA1*, requires further investigation to identify its role to the development of exudative AMD.

This genome-wide association study has demonstrated its power to be effectively and comprehensively in testing and revealing genetic variation across the genome for a role in common disease. The cause of AMD, a multifactorial disease, is best described by the CDCV hypothesis in which multiple modest variants together play a role in developing this common disease. We have clearly identified the involvement of a new variant in *HTRA1* in AMD, adding a new piece to the puzzle of how genetic factors predispose the complex disease. This powerful approach would also facilitate in the identification of

more genetic factors in molecular classification of various subtypes of AMD, such as polypoidal choroidal vasculopathy (PCV) and retinal angiomatous proliferations (RAP).

4.1.1. Limitations and Concerns of Genome-Wide Association Study

Although this study has demonstrated the success and power of genome-wide association study approach in identifying novel susceptible disease locus/gene, there are a number of limitations that need to be addressed. Genetic heterogeneity of the sample population is one of the important factors to be considered for spurious association. Population substructure can lead to false conclusions so as racial difference. It is essential to replicate association findings in other ethnic population to confirm any conclusions, especially when only modest effects of the variants are observed. MAF of Y402H at *CFH* is extremely low (0.04) to detect any significant association to AMD in Chinese and Japanese populations. The observed high prevalence of the risk C allele of Y402H at *CFH* in the Caucasians revealed the effects of racial difference. Most common, complex genetic disorders do not reflect a single model, but result from contribution of various mechanisms. Under these circumstances, the extent of common variants relating to the common, complex disorder is based not only on the disease itself and the relevant genes but also on the particular population that are studied. To further support the findings of this study, replications need to be done in other ethnic populations and was successfully done be subsequent investigations by other researchers as mentioned earlier.

There is a need to adjust the level of significance to account for the large number of variants that are tested and use a highly stringent criterion for concluding the result. This

is necessary for eliminating any spurious association due to inflation of type 1 error in multiple testing in a genome-wide association study.

Another key component for genome-wide association study approach is sufficient sample size so as to attain enough power to detect genes of modest risk to the disease. Power studies have shown that at least 2000 to 5000 samples for both cases and controls are required for genome-wide association study with modest risk (Odds Ratio about 1.2-1.5) to successively identify the disease-predisposing variants (Pfeiffer and Gail, 2003;Risch and Merikangas, 1996). The present study has only worked on 100 cases and controls and was able to detect the strong association. This was mainly due to the distinctively high prevalence of the risk C allele found in the disease (Minor allele frequency [MAF]=0.59) and its strong association to the risk of disease (LOC387715-rs10490924: OR_{hom}=11.14; 95%CI [4.83-25.69]). In a general modest associated variant, a large recruitment of cases and controls will be necessary. The recruitment of large cohorts for genome-wide association study is a challenge for collecting cases with consistent clinical phenotype. To recruit a large number of pedigrees in linkage studies, each containing multiple affected individuals especially for diseases of old age such as AMD is nevertheless as difficult as the recruitment of large cohorts.

4.2. HTRA1 Genotyping

4.2.1. Association and Haplotype Analysis

Age-related macular degeneration is the leading cause of visual impairment in the elderly and a major cause of blindness in the developed countries. Recently, *CFH* was identified as the major associated gene leading to the advanced stage of the disease. In this study, *HTRA1*, being suspected to be associated with exudative AMD, was screened for its association. Two variants in the promoter and two variants in the exon 1 were identified to be associated with exudative AMD. Carrier of the risk allele (AA) of the most significant SNP, rs11200638, being at the promoter increased the risk to exudative AMD with an OR of 7.9. This significant finding was replicated by another case control study of exudative AMD patients in Japanese population that demonstrated the association of *HTRA1* promoter polymorphism, rs11200638, to exudative AMD with an OR of 10.1 (Yoshida et al., 2007). Consistent significant association of rs11200638 to risk of the two advanced forms of AMD, GA and exudative AMD, was also identified in subsequent study in Caucasians (Cameron et al., 2007). Replications of the association of rs11200638 at *HTRA1* with AMD in other ethnic population further support the significance of *HTRA1* in AMD development and progression.

HTRA1-rs11200638 risk allele is common in the Chinese population, with 20% of controls having homozygous risk allele (Table 3.11a). This group of controls carrying two copies of the *HTRA1* risk allele has higher risk to develop AMD at a later period of time and will require close monitoring of any signs of manifesting advanced AMD for early detection and treatment.

rs11200638 lies 512bp upstream of the *HTRA1* transcription start site and resides within

putative binding sites for the transcription factors, adaptor-related protein complex 2 alpha (AP2α) and serum response factor (SRF) (Dewan et al., 2006). The presence of the risk allele was predicted to alter the binding affinity of AP2α and SRF, which is expected to disturb the expression of *HTRA1* in certain extent. This genotype-driven expression pattern was demonstrated by the observation of an increase trend of luciferase expression in *HTRA1* promoter-driven constructs in the AA risk genotype-bearing constructs than the GG carriers in transfected RPE and HeLa S3 cells (Dewan et al., 2006). Another study has shown a stronger immunohistochemistry results in the drusen and along Bruch's membrane of AMD patients and an elevated expression of *HTRA1* mRNA and protein to be associated with the AA risk genotype carrier of AMD patients (Cameron et al., 2007;Yang et al., 2006). These subsequent findings supported our result that AA risk carrier of rs11200638 at *HTRA1* in the promoter was associated with risk of exudative AMD by upregulating the expression of *HTRA1*. The role of genotype-driven increase expression of *HTRA1* leading to the development of exudative AMD remains speculative. The possible function of *HTRA1* will be discussed in the following section so as to postulate its possible role to the pathogenesis of exudative AMD.

4.2.2. HTRA1

HtrA1 (High temperature requirement) is a member of heat shock serine proteases and is upregulated by cellular stress like oxidative stress in the course of degeneration. It is involved in arthritis, cell growth, unfolded stress response, programmed cell death (apoptosis), and aging. It belongs to HtrA protein family and is a secreted protein. It is

characterized by catalytic activity and reversible zymogen activation (Ehrmann and Clausen, 2004). Its N terminus is homologous to Mac25 (the Insulin-like growth factor-binding domain, IGFBP) with a conserved Kazal-type serine protease inhibitor (KI) motif, and C-terminal has a PDZ domain (Figure 4.2). This Mac25/IGFBP is a follistatin-like protein (also, activin-binding protein) involving in proteolytic activity in releasing IGF from IGF-binding proteins (Zumbrunn and Trueb, 1996;Zumbrunn and Trueb, 1997). It has also been demonstrated to have tumor suppressive activity by suppressing growth in several tumor cells, such as sarcoma cells, cervical carcinoma cells, and embryonic carcinoma cells (Kato, 2000). This suggests that HtrA1 might be involved in cell growth regulation by modulating the response to the growth factors targeted by the mac25 domain. The linker region and the SP domain bind to transforming growth factor Tgfβ family members, regulating the extracellular matrix (ECM) (Kresse and Schonherr, 2001;Oka et al., 2004). Secreted HtrA1 may bind to ECM around the cell, become fully active and subsequently, inhibit Tgfβ signaling arising from the proteolytic activity of HtrA1.

The diverse multiple roles of *HTRA1* have been evaluated from numerous studies, yet still no specific biological functional can be concluded. The knowledge of the differential cell and tissue-specific pattern of expression of *HTRA1* in normal human tissues reflect some insights of its possible role in the etiology of exudative AMD. Its observation of down-regulation in several human tumors, such as ovarian cancer (Bowden et al., 2006;Chien et al., 2004;Shridhar et al., 2002) and melanoma (Baldi et al.,

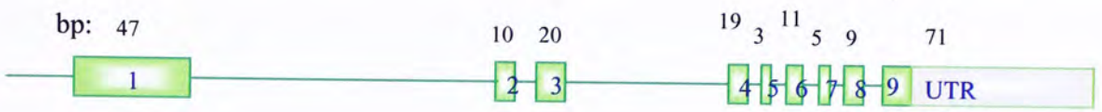
Figure 4.2 Schematic representation of the protein domain of HTRA1

(a) **Genomic view**

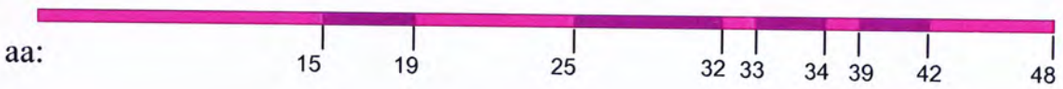
(b) **cDNA view**

(c) **Protein domains of HTRA1**

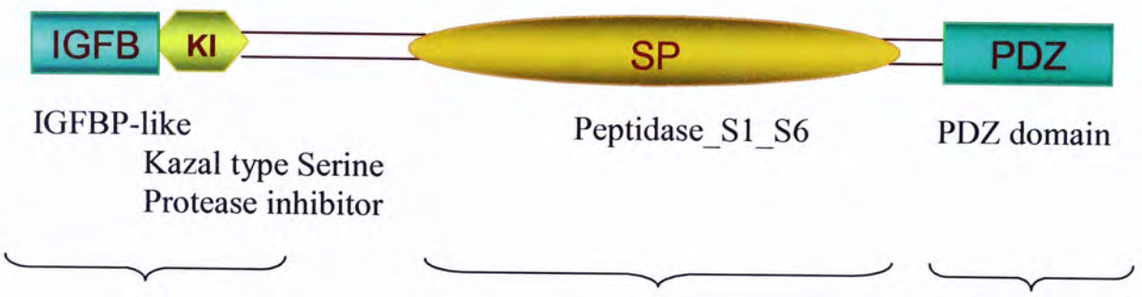
(a)



(b)



(c)



- Bind to IGF-I, IGF-II
- Follistatin-like protein
- Cell growth regulation

- Highly conserved trypsin-like catalytic domain
- Binds to Tgfβ
- Proteolytic activity

- Mediate protein-protein interactions

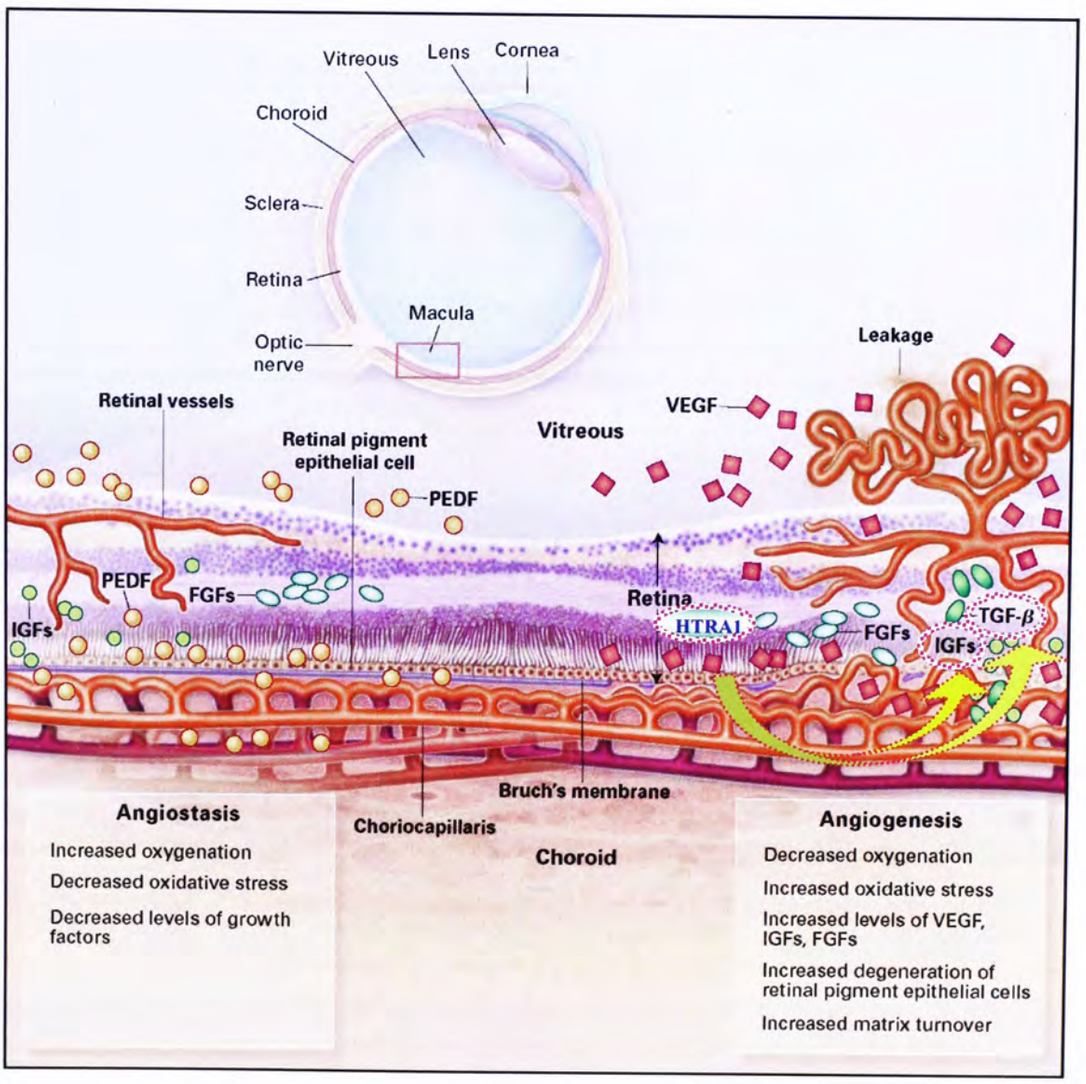
2002) suggested its possible role in cell homeostasis. Another study reported an increase expression of HtrA1 inhibiting in vitro proliferation and in vivo tumor growth, suggesting tumor suppressor function of HtrA1 (Baldi et al., 2002). Some data also suggested that HtrA1 regulating cell death involving in chemotherapthy-induced apoptosis (Chien et al., 2006). Many other reports proposed the secretary function of HtrA1 to be involved in the extracellular space. Elevated synovial HtrA1 levels detected from rheumatoid and osteoarthritis patients resulted in the specific induction of matrix metalloprotease 1 (MMP-1) and matrix metalloprotease 3 (MMP-3) expression (Grau et al., 2006;Hu et al., 1998;Tsuchiya et al., 2005). The cleavage of extracellular proteins, such as fibronectin, by HtrA1 stimulated MMP3 expression suggested this protease might be playing a role in extracellular matrix remodeling. Having extracaelluar localization, HtrA1 was also found to be highly secreted from human mast cells (Gilicze et al., 2007). Contrasting to previous findings that reported an inhibitory effect of HtrA1 on Tgfβ depending on its serine protease activity (Oka et al., 2004), the Tgfβ-inhibitory activity of HtrA1 did not inhibit mucosal mast cell differentiation. Upregulation of HtrA1 expression was also found in the uterus and trophoblast (De et al., 2004;Hu et al., 1998;Nie et al., 2006;Nie et al., 2003) during placental development, suggesting its possible role in placentation.

There is strong evidence implicating the susceptibility of *HTRA1* in the risk of not only in exudative AMD but also in other advanced type of AMD. The observed higher expression of HtrA1 in arthritis individuals coincides with the same condition found in homozygous risk allele carrier in exudative AMD; hence, suggest a clinical history of

arthritis can be relevant. The proposed interaction of HtrA1 with growth factors may be important. HtrA1 may increase the amount of circulating IGF by cleavage from IGF-binding proteins at its N-terminus. An increase of circulating IGF may result in an increase in vascular endothelial growth factors, which are markers for AMD susceptibility (Bhutto et al., 2006;Churchill et al., 2006;Tong et al., 2006a) and therapeutic target for exudative AMD treatment (Gehrs et al., 2006;Tong et al., 2006b). Inhibition of Tgfβ, a potent antiangiogenic molecule, adds further support to HtrA1's role in neovascularization as observed in exudative AMD (Oka et al., 2004). Similarly, inhibition of TGF-mediated homeostatic functions observed in osteoarthritis may have implications in the atrophic development observed in early and dry AMD.

The diverse functions of HtrA1 revealed from numerous studies suggesting its proteolytic role in releasing IGF from IGF-binding proteins, its interaction with extracellular targets on C-propeptides of fibril-forming types I, II and III procollagens (Murwantoko et al., 2004), fibronectin (Grau et al., 2006) or proteoglycans such as aggrecan, decorin, fibromodulin and soluable type II collagen (Tsuchiya et al., 2005), resulting in important function in extracellular matrix remodeling. HtrA1 appears to have dual roles. With its upregulation being switched by stress, it acts as a promoter of angiogenesis via IGF and TGF pathways giving rise to the development and progression to exudative AMD. In addition, overexpression of HtrA1 driven by the homozygous risk A allele at the promoter may alter the integrity of Bruch's membrane by extracellular matrix remodeling, favoring the invasion of choroid capillaries as occurs in exudative AMD (Figure 4.3). Further functional investigation is necessary to characterize this

Figure 4.3 Schematic diagram showing neovascularization due to the growth of choroidal blood vessels into the degenerating layer of retinal pigment epithelial cells. (Diagram was obtained and modified from King and Suzuma, 2000)



- Genotype-driven over-expression of HTRA1
- HTRA1 cleaves IGFBP and further increased levels of IGF
- Further induced angiogenesis
- HTRA1 binds to TGF□, induced extracellular remodeling for invasion of vascularization system

proposed mechanism in genotype-driven neovascularization observed in exudative AMD.

4.2.3. Gene-Environment Interaction

Smoking is a known risk factor of AMD. In this study, 85% of the 346 patients had information on smoking history. The frequencies of the risk A allele of *HTRA1*-rs11200638 associated with AMD-risk in smokers (36.6%) and non-smokers (36.2%) were very similar whereas that with controls in smokers (18.5%) and non-smokers (25.9%) were two-fold lower as shown in Table 3.11a (on page 73). Smoking appeared to be independent of the rs1120063 variant in *HTRA1* with no significant interaction. Numerous studies had reported smoking to be associated with risk of advanced AMD independently with no interaction with other susceptible genes studied, LOC387715, *APOE,* and *CFH* (Francis et al., 2007;Schmidt et al., 2005;Scott et al., 2007;Seddon et al., 2006b). Only one recent study had reported smoking as a modifier of LOC387715, an AMD-susceptible gene (Schmidt et al., 2006). Whether smoking and *HTRA1* interacted or not resulting in developing AMD, this would require sample sizes with smoking status to precisely identify the specific interaction estimates. Smoking has been reported to promote inflammatory activity with observable alteration of plasma *CFH* levels (Esparza-Gordillo et al., 2004;Seddon et al., 2006b). It is necessary to investigate on the targets of environmental factors (smoking) that modify gene function (*HTRA1*) which will better assess the effect of environmental exposure on *HTRA1*.

In this study, the large increase of PAR percentage estimates in smoking (12%) and

HTRA1 (53%) to the joint effect (68.7%) (as shown in Table3.11b on page 73) indicating smoker carrying the risk allele will be at high risk of developing the disease. High prevalence of the risk A allele at *HTRA1*-rs11200638 in exudative AMD (MAF=73.2%) and controls (MAF=44.4%) were found. This reinforces and motivates the necessity of modifying our lifestyle in quitting smoking and having regular eye examinations, especially for those high risk controls, being homozygous carrier of the variant, who are at higher risk in developing AMD later.

4.2.4. Gene-Gene Interaction

CFH Y402H is associated to risk of AMD by altering the complement system and promoting inflammatory activity that leads to the development of drusens found in AMD patients. CFH-rs800292 was used to examine gene-gene interaction between *CFH* and *HTRA1*. The two susceptible genes acted independently with no interaction was identified. The risk of CFH-rs800292 to exudative AMD (OR=2.14; 95% CI [1.18-3.86]) was similar to that of smoking (OR=1.76; 95% CI [1.11-2.80]) while the risk of HTRA1-rs11200638 to exudative AMD was substantially higher (OR=7.94; 95% CI [3.95-15.97]) (as shown in Table 3.12b on page 76). These data supported the stronger impact of *HTRA1* to exudative AMD than smoking and *CFH* in this study. The joint risk of *CFH* and *HTRA1* to exudative AMD was 23.33 with 95% CI being 2.49-218.24 (Table 3.12). *CFH* and *HTRA1* appeared to be involved in different pathway in the development of exudative AMD. A recent study has reported immunohistochemical detection of HtrA1 in drusen of GA patients (Cameron et al., 2007). Evidence has shown the Y402H at *CFH* being associated to risk of AMD in both GA and exudative AMD.

This genotype data together with the observed expression of HtrA1 in drusen of GA patients suggest that *CFH* and *HTRA1* contribute to the development and progression of advanced AMD. Although our data has shown independent effect of the two genes on exudative AMD, further study of interactions in prospective studies with larger sample sizes are required to confirm the findings.

The high prevalence of the joint *CFH*-rs800292 and *HTRA1*-rs11200638 variants (prevalence of homozygous for either or both variants = 82.2%; Table 3.12a on page 76) and their strong association to exudative AMD (Joint OR = 23.33) raises the possibility of population genetic test of the joint-genotype in risk prediction. The availability of the joint variants' genetic test may help to predict the risk of developing exudative AMD. The potential benefits of identifying high risk, pre-symptomatic subjects would allow insight into the earliest manifestations of disease and allow trials of preventive treatment in high-risk groups. In addition, knowledge of the substantial risk of developing AMD in the high-risk subjects will provide motivation for them to aware of their lifestyle such as stop smoking, lose weight and have regular eye examinations. Recently, variant of transcription factor 7-like 2 (*TCF7L2*) gene has been identified to confer risk of type II diabetes giving rise to the opportunity in launching the first predictive genetic testing on one's risk for developing diabetes in April, 2007 (Grant et al., 2006;Humphries et al., 2006;Zhang et al., 2006). Although genetic testing will only suggest the perspective risk to the disease, present findings favor the consideration of joint-loci predictive genetic test of AMD in which early detection of high-risk groups would result in a more

favorable outcome. This would help in early detection of manifestation of the disease and immediate treatement to delay the disease from progression.

Chapter 5

Conclusions and Future Aspects

The work of two attempts, one by genome-wide linkage disequilibrium association study and the other by candidate gene screening, to search for the genetic basis of AMD was described in this thesis. In the genome-wide association study, a single SNP, rs10490924, on LOC387715 at chromosome 10q26 was identified to be significantly associated with exudative AMD (p-value = 4.1×10^{-12}). The odds ratio conferring the disease was 11.1 with 95% CI being 4.83 to 25.69. The high PAR of 86% implicates the extreme high prevalence of the risk allele that exists in the Chinese population. Haplotype analysis further confirmed the significance of the SNP. Another interesting SNP in perfect linkage disequilibrium with rs104090924, rs11200638 at *HTRA1*, was identified to confer high risk to exudative AMD, with an OR of 10.0 and PAR of 84%. Our success in obtaining a new AMD susceptible variant verifies the powerful approach of genome-wide association in studying the genetics of complex diseases. Consistent with our experiences, recently a variant in the *TCF7L2* gene and *CDKAL1* gene were identified to be associated with type II diabetes utilizing the same genome-wide association study approach (Owen and McCarthy, 2007;Sladek et al., 2007;Steinthorsdottir et al., 2007).

LOC387715 is a hypothetical gene with no known biological function. It has only been reported to express in the placenta and sparsely in the retina. *HTRA1*, residing adjacent to

LOC387715, was chosen for investigation of its association with exudative AMD. We found no disease causing variants. Instead, variants strongly associated with exuative AMD are identified. Variants in the promoter and the first exons of *HTRA1* were identified to be strongly associated to risk of exudative AMD. Variants in the exons are synonymous changes. An interesting variant in the promoter at rs11200638 was found to be significantly associated with exudative AMD. These variants are in perfect linkage disequilibrium. Homozygous risk allele carrier is at 7.64-fold (95% CI of 3.98-14.66) higher risk to disease (p-value = 1.1×10^{-10}). The observed high PAR of 55% suggests that as much as half of the population carrying the homozygous risk allele will develop the disease. Evidence has shown our success in identifying an important variant in the genome being associated with exudative AMD. This *HTRA1* variant with rs11200638 is the second AMD susceptible gene discovered. The first AMD susceptible gene, *CFH*, is only at 2.14-fold (95%CI 1.18-3.86) risk to exudative AMD. The *HTRA1* rs11200638 exerts a much higher impact than the *CFH* variant rs800292 in our Chinese exudative AMD patients. The SNP at the promoter of *HTRA1* affects the risk of a distinct AMD phenotype, exudative AMD, which has shown higher prevalence in Asians than in Caucasians. Consistent replication of its association to exudative AMD and other advanced type of AMD, geographic atrophy convincingly reinforced the importance of the *HTRA1* variant in the etiology of AMD (Cameron et al., 2007;Yang et al., 2006;Yoshida et al., 2007). *CFH* is involved in the alternative complement pathway altering the immune response results in the abnormal deposition of drusen as observed in GA patients. No definitive biological role of *HTRA1* has been reported (Dewan et al., 2007). Further work should focus on the investigation of the physiological substrates of *HTRA1* in order to reveal its biological functions and

activities and will provide better understanding of the molecular mechanism underlying the role of *HTRA1* in the development of exudative AMD.

Gene-environment and gene-gene interactions in this study showed smoking to be a major environment risk factor to AMD. The risk AA genotype at rs11200638 on *HTRA1* independently increases the risk of exudative AMD. Results of our analysis gave evidence that *CFH* and *HTRA1* acted independently with no interaction in the development of exudative AMD. They appear to be involved in different pathways in the etiology of advanced AMD. Further investigation on the joint mechanisms of the two genes on different subtypes of AMD will assist in rational drug design and effective prognosis and clinical management in the future.

Electronic-Database Information

The URLs for data presented herein are as follows:

D. Clayton's Web site: http://www-gene.cimr.cam.ac.uk/clayton/software/ (for SNPHAP).

M. Stephens' Web site: http://www.stat.washington.edu/stephens/software.html (for PHASE)

GOLD Web site: http://www.sph.umich.edu/csg/abecasis/GOLD/

Haploview Web site: http://www.broad.mit.edu/mpg/haploview/

Haploxt Web site: http://www.sph.umich.edu/csg/abecasis/GOLD/docs/haploxt.html

Ensemble web site: http://www.ensembl.org/index.html

R statistical analysis package: http://www.r-project.org/

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